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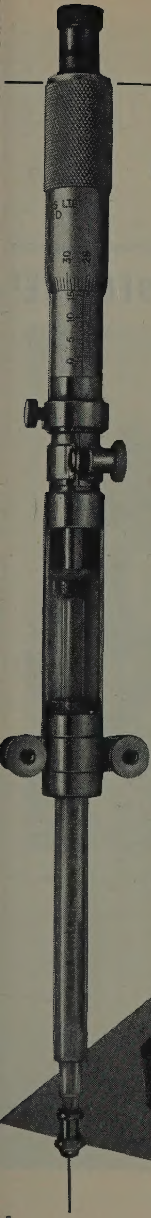

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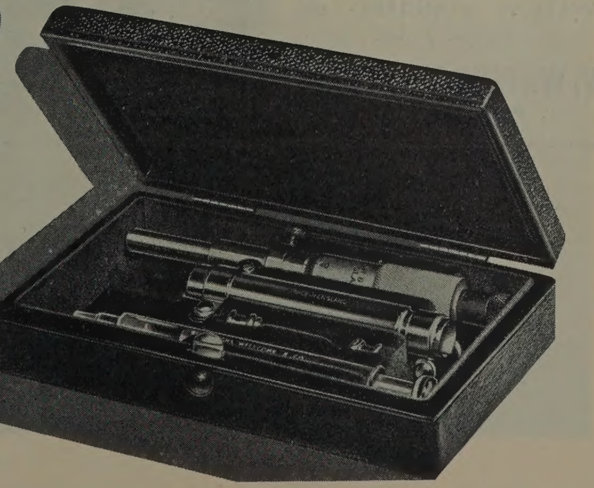

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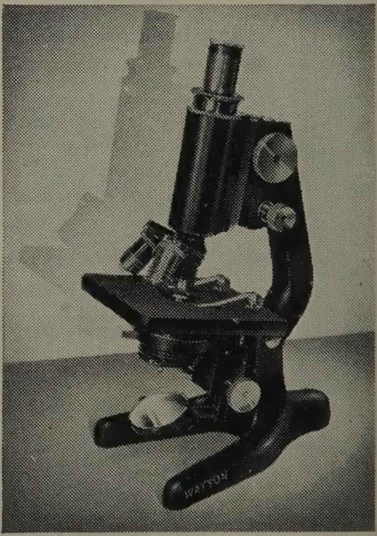

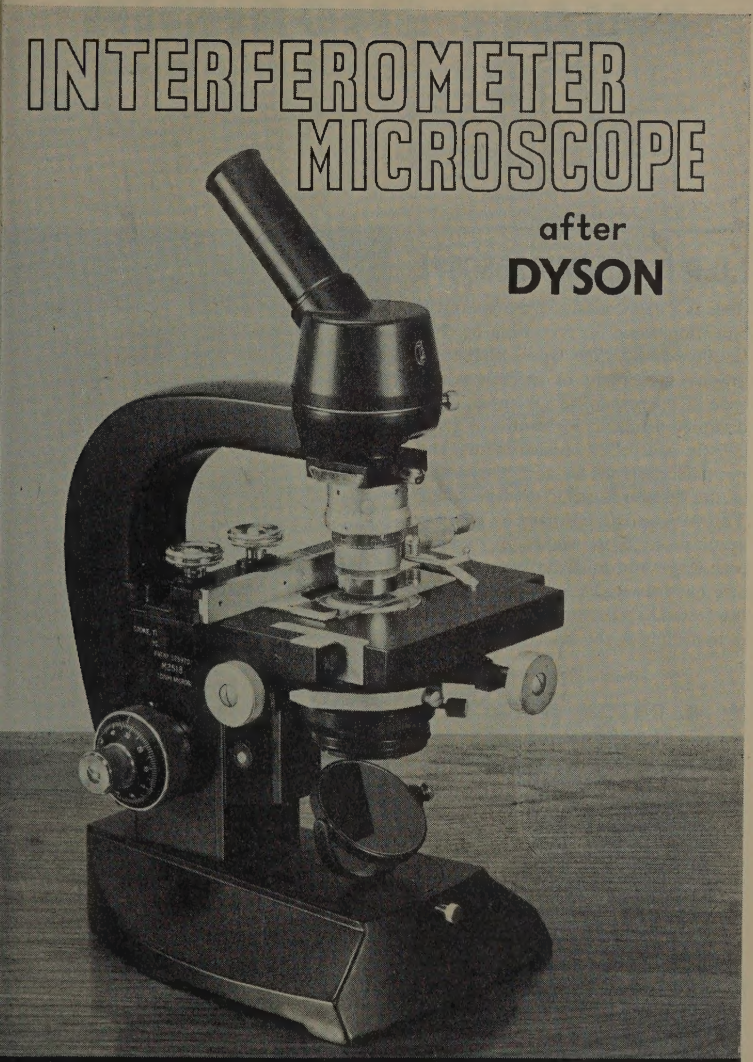

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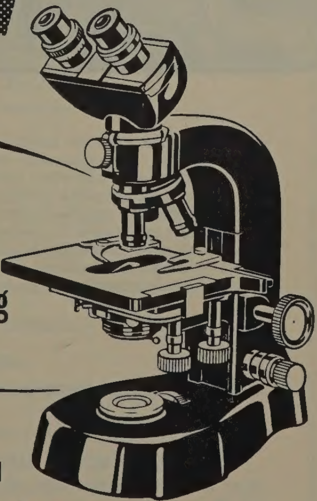

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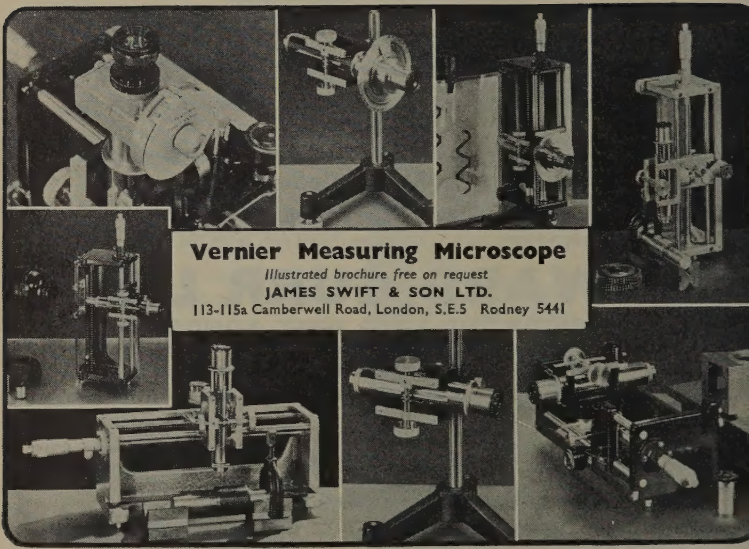

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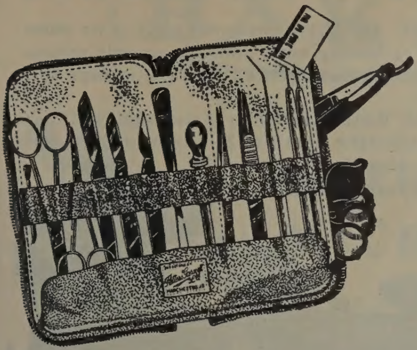

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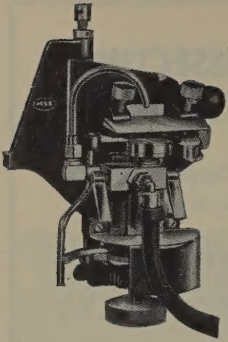

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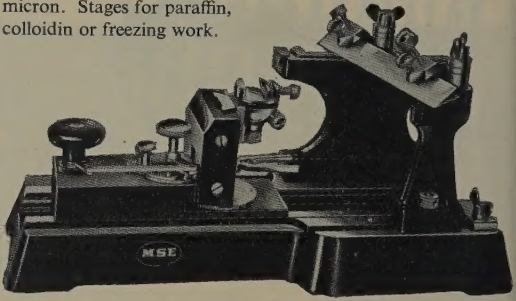

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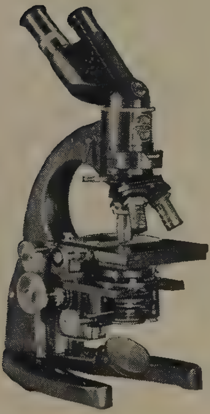

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A Study of Potassium Permanganate 'Fixation' for Electron Microscopy

By S. BRADBURY AND G. A. MEEK

(*From the Department of Human Anatomy, South Parks Road, Oxford*)

With five plates (figs. 1, 2, 4, 5, and 6)

SUMMARY

The action of buffered potassium permanganate as a fixative for electron microscopy has been investigated. Electron contrast has been shown to be produced by the deposition of granular material in the tissue. The particles are about 50 Å in diameter. Actual fixation of the tissue is performed by the dehydrating alcohol.

Histochemical studies have shown that RNA and histones are removed, whereas phospholipid-protein complexes are 'unmasked'. The reaction of the permanganate with unmasked protein gives rise to high membrane contrast in electron micrographs.

INTRODUCTION

THE preparative techniques at present in use for electron microscopical studies of biological materials involve considerable changes in the structure of the cell (Borysko, 1956). Changes introduced by fixation are obviously important and may represent one of the major sources of error in the interpretation of electron micrographs. Baker (1958) has reviewed the problems of fixation as applied to light microscopy, but relatively little has been published concerning fixation of tissues for the electron microscope. It has become accepted that osmium tetroxide is the most suitable substance for preserving cellular structure for study with this instrument. The researches of Palade (1952) and Bahr (1954) have contributed much to our understanding of the action of this reagent. More recently, however, potassium permanganate has been introduced as an alternative fixative for electron microscopy (Luft, 1956). In general, this reagent reveals cytoplasmic structures which are very similar to those found in osmium-fixed material. There are certain differences, such as the increased contrast of membranous structures, and the 'washed out' appearance of the nuclei after permanganate treatment. It seemed advisable to investigate the effect of permanganate in more detail in an attempt to obtain further information about its action on cells and so help the interpretation of the appearances seen in electron micrographs.

It is the purpose of this paper to describe both the experiments which were carried out in order to test the action of permanganate on various gels (representing crude models of protoplasm), and the results of a combined histochemical and electron microscopical study of permanganate-treated tissue.

MATERIAL AND METHODS

For the studies of the action and rate of penetration of potassium permanganate, experiments were performed on 'models' of protoplasm, consisting

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of gels of gelatin, gelatin and albumin, gelatin and globulin, and gelatin with phospholipid. With the exception of the phospholipid gel, these were prepared as described by Baker (1958). 15 g of gelatin were dissolved in 100 ml of albumin prepared by diluting egg white with twice its volume of water. The preparation was kept at 37° C until all the gelatin had dissolved, when it was strained and cast in 30-grain pessary moulds. The resulting plugs of gel were kept in a refrigerator until required for the experiments. The phospholipid was freshly prepared from homogenized egg-yolk by extraction with an excess of ether. After standing in the cold for 48 h, the supernatant liquid was decanted and the phospholipid precipitated by adding twice the volume of acetone. The resulting white precipitate was washed several times with acetone and stored in the dark under acetone. The gel was prepared by adding to 15% aqueous gelatin enough phospholipid previously dried in a vacuum to give a final concentration of 2·5% lipid in the product. The product was then cast in moulds.

For histochemical and electron microscopical studies, pancreas and liver of CBA mice were chosen as standard experimental material. The tissue was fixed with ice-cold 1% buffered (pH 7·4) osmium tetroxide, or in 10% formaldehyde-saline for purposes of histochemical control. Experimental material was treated with ice-cold 1% buffered (pH 7·4) potassium permanganate (Luft, 1956). Dehydration by passage of the material through graded strengths of alcohol was followed by embedding in butyl methacrylate or in araldite for electron microscopy. The histochemical studies were carried out on material embedded in paraffin or gelatin, and in some cases on 2μ sections cut from the methacrylate blocks. Ultrathin (50 to 100 $m\mu$) sections were cut on a Porter–Blum microtome and examined in a modified Siemens Elmiskop I (Meek, 1959), with double condenser (400μ aperture), beam current of 10 μA at 60 kV, and objective aperture of 30 μ.

RESULTS

Before attempting to study the effect of permanganate solution on tissues, data were obtained on its action on artificial models of protoplasm. Observations were made on changes in the size and colour of the treated gels; their stability in warm water was also noted.

Volume changes of gels

Fig. 1 shows a series of photographs of plugs of gel, before and after treatment with fixatives. Gelatin-phospholipid gels are shown in fig. 1, A; gelatin-albumin gels in fig. 1, B. It will be observed that in both cases osmium-fixed gels were darkened and shrunken while those treated with permanganate

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FIG. 1 (plate). A, the appearance of gelatin-phospholipid gels after treatment with potassium permanganate, formaldehyde, and osmium tetroxide; an unfixed gel is included for comparison.

B, gelatin-albumin gels treated with the same reagents as in A.

C, the stability of gelatin-albumin gels to hot water.

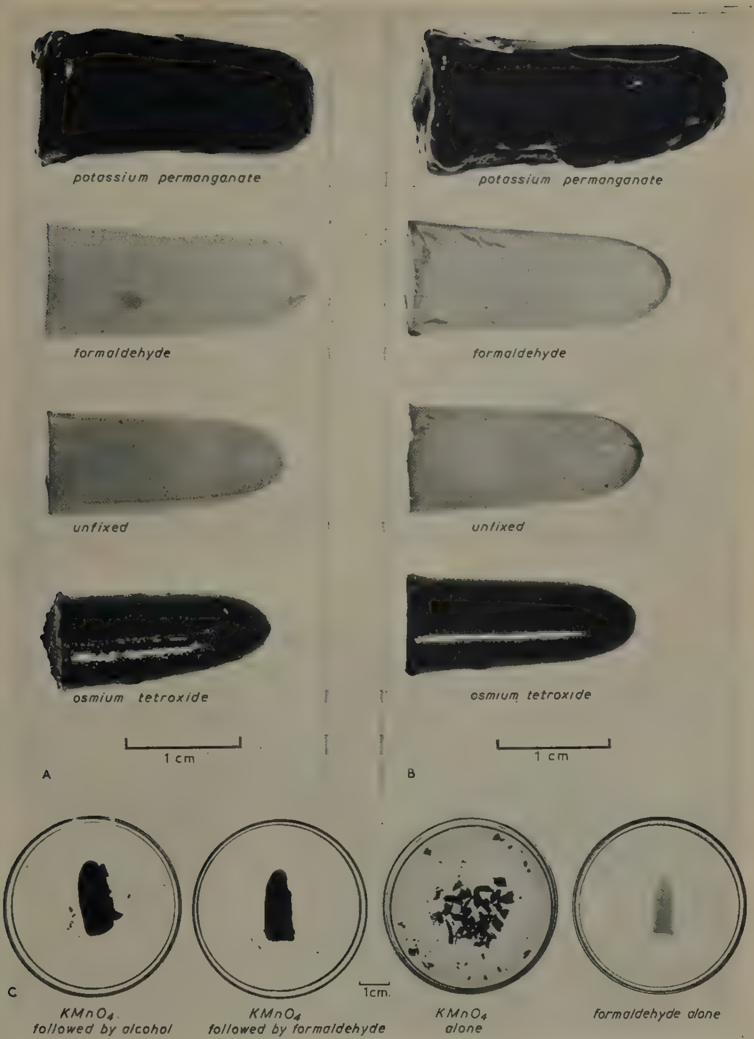

FIG. 1

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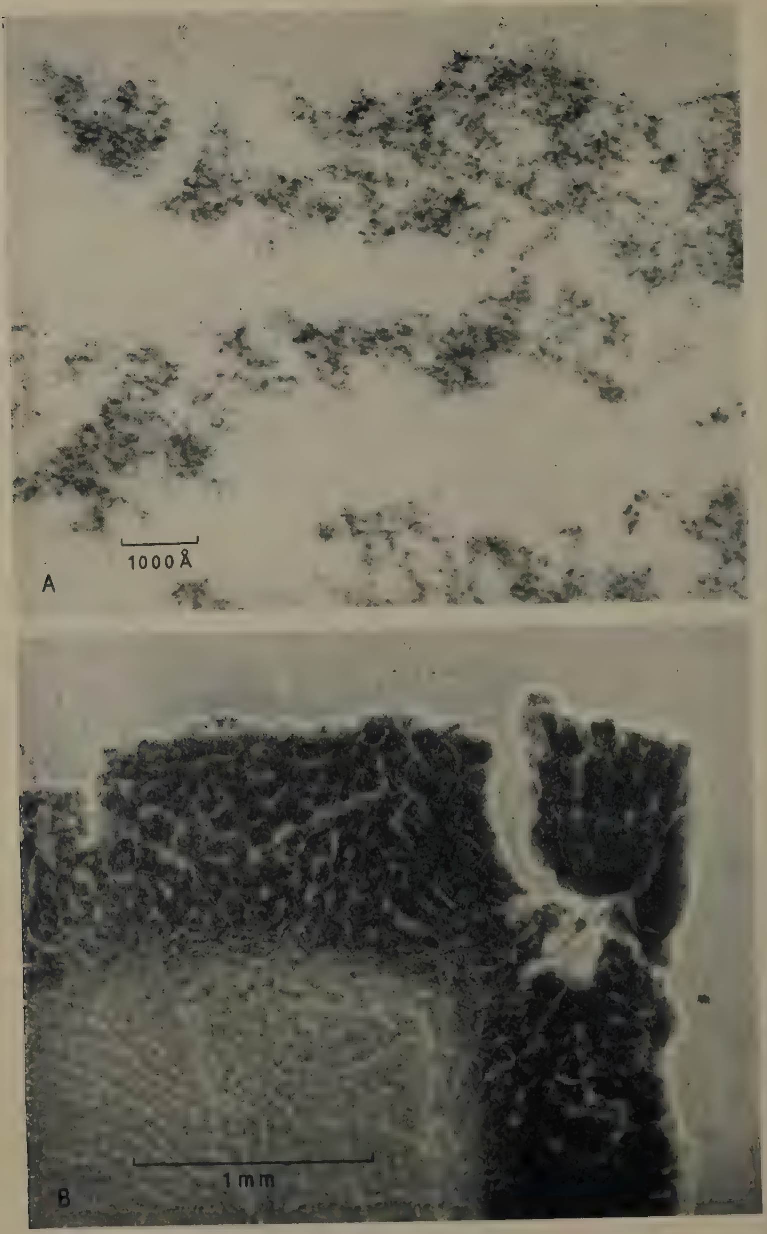

FIG. 2

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were darkened and swollen. The formaldehyde-fixed plugs were slightly swollen but not darkened. Similar appearances were obtained with gelatin alone and with gelatin and globulin. Table 1 gives the percentage volume change of gelatin-albumin and gelatin-phospholipid gels; no measurements were made on the other gels.

TABLE 1

	Percentage volume change	
Treatment	*Gelatin-phospholipid*	*Gelatin-albumin*
$KMnO_4$	+70	+100
HCHO	+70	+70
OsO_4	−20	−20

Stability of gel

Fig. 1, C shows the stability to warm water of plugs of gelatin-albumin gels after the following treatments: permanganate followed by 70% alcohol; permanganate followed by formaldehyde; permanganate alone; formaldehyde alone. It will be observed that the plug treated with permanganate alone disintegrated completely, whereas further treatment with either alcohol or formaldehyde stabilized the form, although incompletely. Formaldehyde fixation alone stabilized the plug completely. A similar result was obtained with the gelatin gel. With the gelatin-globulin gel, it was found that there was partial stabilization of form towards hot water; again, subsequent treatment with formaldehyde or alcohol increased the stability of form.

When the stability of phospholipid-gelatin plugs to warm water was tested, it was found that the alcohol treatment following permanganate no longer rendered the plug insoluble, and it disintegrated within one hour.

In an attempt to find out whether the dark coloration of the gels was due to the reaction of the permanganate with protein, a 2·6% solution of egg albumin was mixed with an equal volume of permanganate solution. There was an immediate colour change, the purple solution becoming brown and turbid, owing presumably to the formation of a very fine precipitate of manganese dioxide, which remained in suspension for at least 24 h. It had previously been noticed that the addition of alcohol to permanganate-treated tissue caused the brownish colour to darken to black, perhaps by reduction of the excess permanganate in the tissue by the alcohol. In order to find out whether such a reduction could take place, potassium permanganate solution was added to absolute alcohol in a test tube. There was an immediate deposit of a coarse dark-brown precipitate, indicating that these substances do in fact react with each other. Since acetone was found not to reduce permanganate,

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FIG. 2 (plate). A, an electron micrograph of a gelatin-albumin gel. The gel was treated with permanganate and embedded in butyl methacrylate.

B, a light micrograph of a 2μ section of liver embedded in methacrylate after treatment with permanganate; the darkening represents the extent of penetration of the reagent.

the possibility of minimizing such precipitation in the tissues by the use of acetone as a dehydrating agent was considered. When this was tried on gels which had been treated with permanganate and with osmium tetroxide, it was found that they became very hard and much shrunken. A similar result was obtained when the gels were soaked in a solution of permanganate in acetone. There was, however, no gross blackening of the gels, and no reduction of the permanganate remaining in the solution. Tissues treated in the same manner and studied with the electron microscope showed a good preservation of cytoplasmic structure. There appeared to be a little less general precipitation of electron-dense material in the ground cytoplasm, but the effect was not striking. The gross darkening observed with potassium permanganate thus seems to be largely due to the deposition of reduction products of the permanganate, possibly in a granular form. Examination of sections of gels and of tissues treated with permanganate and mounted unstained for examination with the optical microscope failed to reveal any such granular deposit, although their brown colour was very obvious. Pieces of the gelatin-albumin gel treated with permanganate and then prepared for electron microscopy by the standard procedure, when examined at a magnification of × 100,000 were clearly seen to consist of separate granules of electron-dense material, about 50 to 100 Å in diameter (see fig. 2, A). Pieces of a similar gel fixed in osmium tetroxide and examined at the same magnification were found to be structureless.

Penetration of permanganate

Medawar (1941) directed attention to the study of the rate at which fixatives penetrate cells and tissues. Baker (1958) used the coagulation of gels by certain fixatives as an index of penetration and extended Medawar's work. In our experiments it was found that the limit of penetration of the permanganate could be measured by observing the extent of the darkening which it produced. Observations made at intervals during a period of 64 h were plotted and are shown in fig. 3, together with the rate of penetration of hydrochloric acid for comparison, measured by observing the extent of coagulation. From the results of Medawar, it is apparent that fixatives entering either a tissue or an artificial gel model of protoplasm obey the laws of diffusion; the relationship between distance of penetration and time is given by the expression $d = K\sqrt{t}$, where d is the distance of penetration in a time t. K is a constant which has a different value for each fixative fluid. Baker (1958) plotted d against $\sqrt{t}$ and found a linear relationship for most of the fixatives studied. Osmium tetroxide is the only fixative in general use which differs significantly from this; here the fixed gel appears to offer some resistance to the entrance of the fixative, so that although for periods of up to 16 h, K has a value of 1, after this there is a falling off to a value of approximately 0·3.

Our results indicate that the rate of penetration of potassium permanganate is very similar to that of osmium tetroxide. For periods up to 9 h the K value is 1, but after this time the value falls off progressively, to 0·65 at 64 h. It seems as though a similar barrier exists in the gel to the entrance of fresh permanganate; it may be that deposits of reduction products of the permanganate act as a physical barrier to entrance of the reagent. When a rather homogeneous tissue such as liver is placed in the permanganate, the darkening produced enables a direct estimate of the distance of penetration to be obtained, as in the case of the gels. Fig. 2, B is a low-power light micrograph of mouse liver soaked in a 1% solution of potassium permanganate for 1 h. It is clear that there has been very even penetration; the tissue subjected to the action of permanganate appears dark, whilst the inside of the block (fixed in effect by the first dehydrating alcohol) appears light in colour. There is a sharp boundary between these zones and it was found that the cells which lie on each side of this 'penetration boundary' showed striking variations with histochemical tests to be described. It was also noticed that the cells on the outside of the block were very poorly preserved, showing large cracks and swelling, with gross deformation of the tissue and cell outlines with both light and electron microscopy (fig. 4, B). The optimum fixation was found in the intermediate zone, between the outermost cells and the 'penetration boundary'.

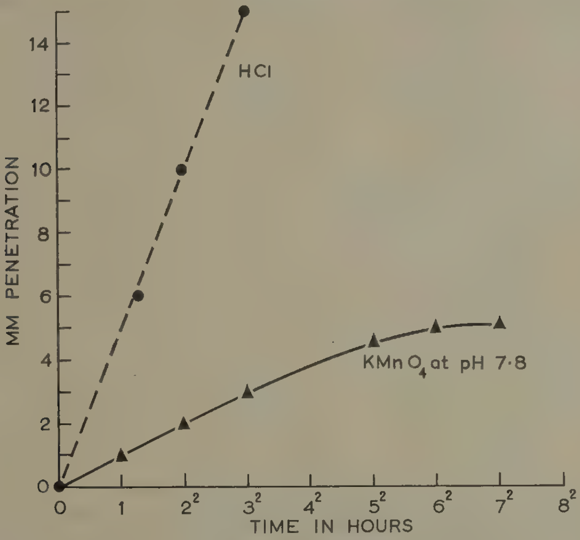

FIG. 3. The rate of penetration of potassium permanganate and hydrochloric acid into a gelatin-albumin gel.

Histochemistry

Before carrying out dehydration for histochemical studies with the light microscope, an attempt was made to remove the excess permanganate from the tissue by leaving the material in gently running water for periods up to

12 h. This procedure invariably led to the total disintegration of the tissue, as in the case of gels; passing the tissue directly into the first dehydrating alcohol (70%) preserved the form of the material. It was necessary to change the alcohol at intervals until no further discoloration of the alcohol was seen.

The histochemical tests were directed in the first instance to checking the findings of Luft (1956), who reported that the tissue retains sufficient deoxyribonucleic acid (DNA) after permanganate treatment to give a positive Feulgen reaction, and that the particles of Palade appear to be absent from the membranes of the endoplasmic reticulum, which would imply an absence of cytoplasmic basiphilia.

Nucleus. The Feulgen reaction was applied to both paraffin sections of permanganate-treated material and also to sections of material embedded in butyl methacrylate which had been soaked for 1 h in ethylene dichloride in order to remove the plastic. Hydrolysis was carried out in N hydrochloric acid at 60° C for a period of 20 min; the sections were then placed in Schiff's reagent for 90 min. It was found that the nuclei gave a positive reaction; the intensity and coloration were not as great as those in tissues which had been fixed in Zenker's fluid. It may be that some of the DNA in the nucleus was removed by the permanganate or subsequent treatments, or, if it was preserved, some change had occurred which prevented it from responding so readily to the hydrolysis. Periods of hydrolysis exceeding 20 min were not found to increase coloration. It seems from this that the nuclei do in fact retain a considerable proportion of their DNA after treatment with potassium permanganate. Electron micrographs of permanganate-treated material show decreased electron density in the nuclei compared with material fixed in osmium tetroxide (Barer, Joseph, and Meek, 1959 *a*, *b*). It was at first supposed that this difference could be directly attributed to the loss of DNA from the permanganate-treated material, but this does not seem to be borne out by the results of the histochemical studies. Alternatively, permanganate treatment might reduce the basic protein content of the nuclei. The tests for proteins are not very satisfactory; in particular, there do not seem to be any specific tests for the basic proteins—histones and protamines—which form the major protein components of the nucleus. It is well known, however, that the histones are very rich in the amino-acid arginine, for which the Sakaguchi reaction provides a reliable histochemical test (Baker, 1947). When this reaction was applied to osmium-fixed and to permanganate-treated pancreatic exocrine cells, a striking difference was observed in the reactions of the nuclei. In the material fixed in osmium tetroxide, the ground cytoplasm gave a positive reaction, but the nuclear reaction was much stronger. In the permanganate material, on the contrary, only the cytoplasm gave a faint coloration. This suggested that there was in fact some loss of arginine from the permanganate-treated material and that this might account for the observed differences in the electron micrographs. It is necessary to stress the need for caution in interpreting these tests. Since many of these reactions were applied to material fixed in fluids other than those specified by the originators of the

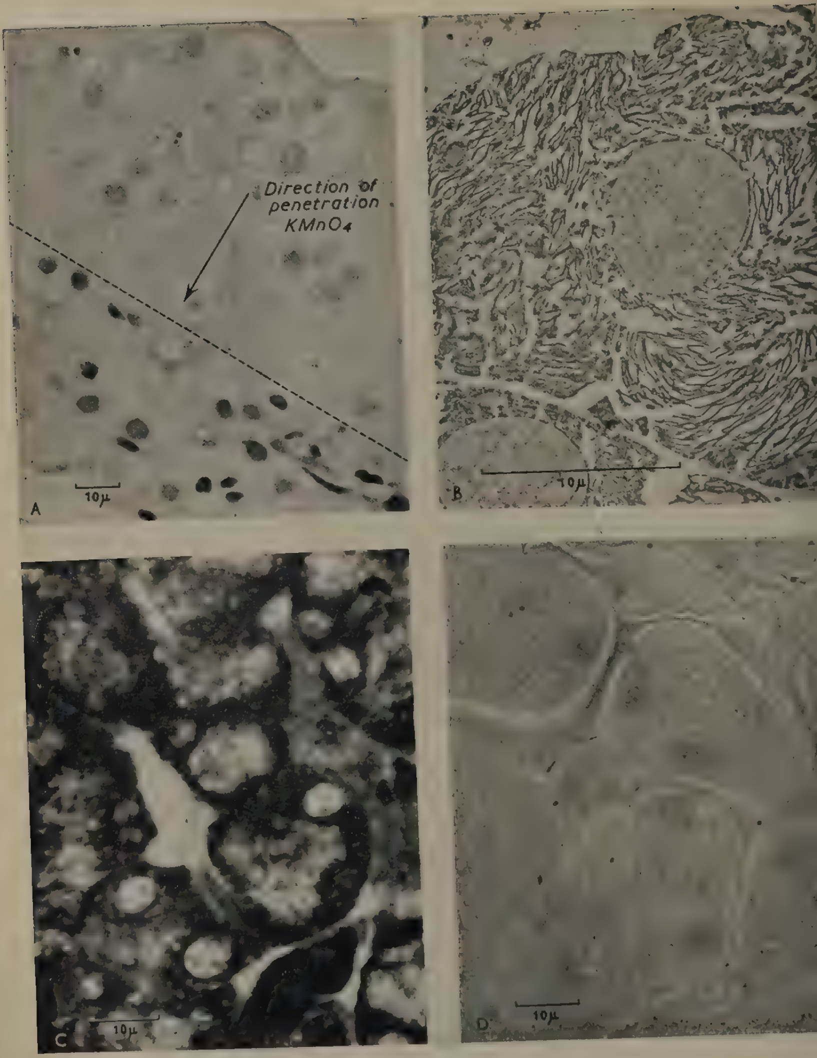

Fig. 4

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tests, their value as strict histochemical tests is diminished. Nevertheless, the localization of substances in the exocrine cell of the pancreas corresponds very well in both the osmium-fixed material and in the control tests applied to cells fixed in the correct reagents.

Two other techniques were used in an effort to obtain further evidence concerning the presence or absence of basic proteins after permanganate treatment. The acid ponceau technique (Hyden, 1943), depending upon the ability of the free amino-groups of lysine and arginine to bind this dye in acid solution, gave no clear-cut results when applied either to our permanganate-treated material or to osmium-fixed tissues. The fast green FCF method (Alfert and Geschwind, 1953) was more successful. Although this method does not claim to be a strict histochemical test, it has been shown to colour selectively such compounds as protamine and histone. These authors showed that nucleic acids interfere with selective staining, so that it is necessary to remove them by a preliminary treatment with hot trichloracetic acid before applying the fast green. Fig. 4, A represents a section of pancreas treated with potassium permanganate and stained with fast green FCF. The section passes through the 'penetration boundary', so that the tissue at the upper right of the figure was subjected to the action of permanganate whilst that on the left was preserved by the dehydrating alcohol. It can be seen that the only nuclei which react to this stain are in that part of the tissue which was not affected by permanganate. It therefore seems probable that potassium permanganate does not preserve the basic proteins of nuclei; the absence of these compounds from the preparation is almost certainly responsible for the changed appearance of the nuclei in electron micrographs.

The contrast between nucleus and cytoplasm in such preparations is enhanced. This is due first to the reduced electron density of the nuclear contents, which may be explained by the above observations; and secondly to the greatly increased contrast of the bounding membrane which frequently shows well-defined pores.

Cytoplasm. Mitochondria in permanganate-treated material show well-marked cristae, but the mitochondrial matrix, which has considerable electron density when fixed with osmium tetroxide, appears to be almost wholly removed by permanganate. The zymogen granules, which are the most prominent inclusion in osmium-fixed pancreas cells (fig. 5, A), are also not rendered electron dense by permanganate and appear as lighter areas bounded by an imperfect membrane (fig. 6, A). The membranes of the endoplasmic

---

FIG. 4 (plate). A, a light micrograph of permanganate-treated pancreas, taken across the 'penetration boundary' (dotted line), and stained by Alfert's method for histones.

B, a low-power electron micrograph of a poorly preserved pancreas cell after permanganate treatment. Swelling and cracking has occurred in the vesicles of the endoplasmic reticulum.

C, a light micrograph of pancreas cells stained with thionine. Cytoplasmic basiphilia appears as dark areas.

D, a light micrograph of pancreas cells treated with permanganate and then stained with thionine; there is no cytoplasmic basiphilia.

reticulum also appear more sharply defined as a consequence of the greater contrast between the paired structures and the ground cytoplasm between them. The distance between the pairs of membranes is significantly greater in permanganate material (figs. 5, B; 6, B) than in osmium-fixed controls.

As reported by Luft, electron micrographs of permanganate-treated material show a complete absence of the ribonucleic acid (RNA) granules, which are such a prominent feature of the endoplasmic reticulum after osmium fixation. Our electron microscope observations confirm this finding (see figs. 5, B; 6, B); as these RNA granules are absent from the cell, the cytoplasm should not show its typical basiphilia. In order to check this, sections of both paraffin- and methacrylate-embedded material were coloured with dyes such as basic fuchsine, pyronin, and thionine. After permanganate treatment, there was a total absence of basiphilia (fig. 4, D), although pancreas fixed in osmium tetroxide and in formaldehyde as controls showed the typical concentration of basiphil substance in the cells (fig. 4, C). The application of the pyronin / methyl green test (Jordan and Baker, 1955), with the ribonuclease control (Bradbury, 1956), shows that this basiphilia of the pancreatic exocrine cell may be attributed to the presence of RNA. There was no staining with pyronin / methyl green after permanganate treatment; this result indicates absence of RNA from the cell.

A further characteristic of electron micrographs of permanganate-treated material is the high contrast of the cytoplasmic membrane systems. Since these membranes are generally believed to be composed of phospholipoprotein, it is possible that potassium permanganate acts as an 'unmasking' agent (Bradbury and Clayton, 1958; Clayton, 1959) and reveals both protein (which reduces the permanganate and so increases the electron density) and lipid in those regions of the pancreatic exocrine cell where the endoplasmic reticulum is most highly concentrated. Gelatin sections of pancreas treated with permanganate were tested for lipids by the standard Sudan black technique (Baker, 1949) and for phospholipids by the acid haematein technique (Baker, 1946). The cytoplasm of the basal region of the cell was found to give a strongly positive reaction to both tests; control sections fixed in formaldehyde-calcium showed that this part of the cell does not normally react to either of these tests. Gelatin sections were then made from blocks which had been treated with permanganate and subjected to the solvent action of pyridine at 60° C; this is known to remove all free lipids. After this procedure, the cells no longer gave a positive reaction to these tests.

The disintegration of permanganate-treated tissues on washing with water which has already been described might be caused by the failure of the reagent to stabilize the mucopolysaccharide ground substance of the intercellular

---

FIG. 5 (plate). Electron micrographs of A, a pancreas cell fixed in osmium tetroxide, embedded in butyl methacrylate. Note the nucleus (*n*), endoplasmic reticulum (*er*), mitochondria (*m*), and zymogen granules (*z*).

B, the endoplasmic reticulum of the osmium-fixed cell.

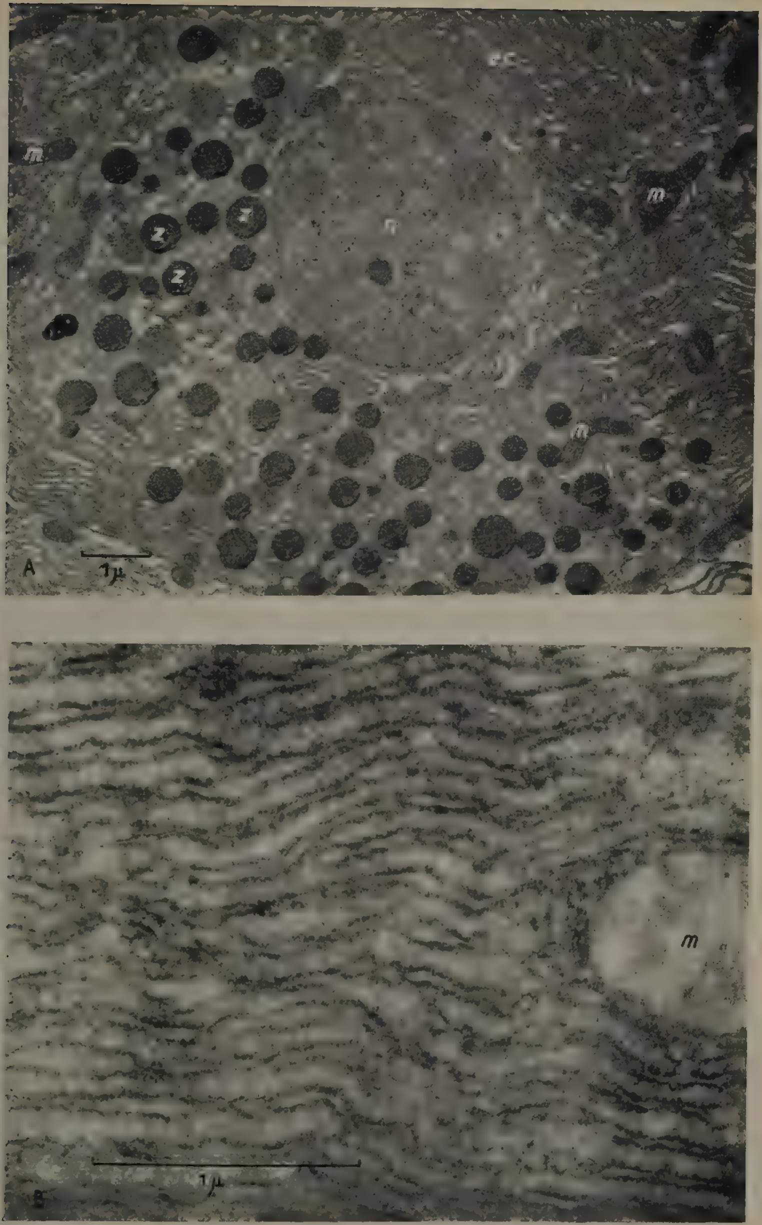

FIG. 5

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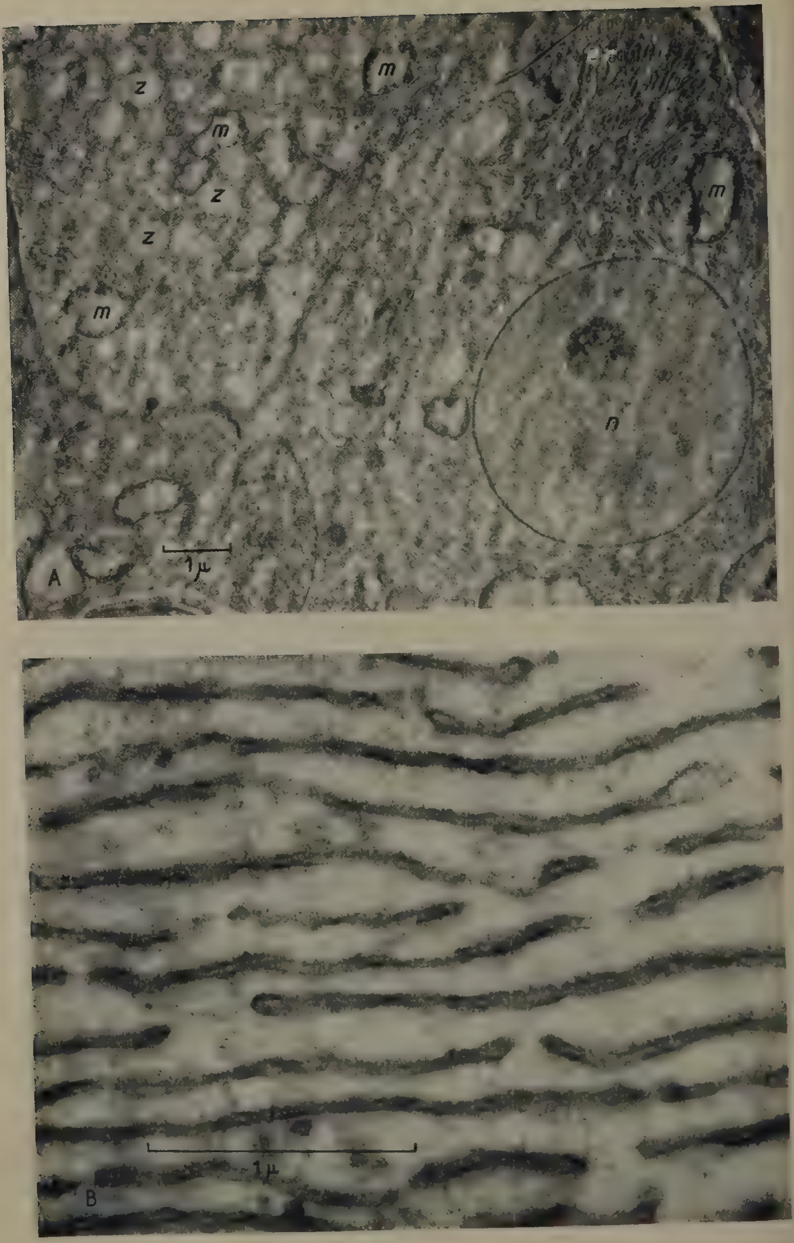

FIG. 6

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connective tissue. Sections were accordingly subjected to the periodic acid/Schiff (PAS) technique, which reveals the sites of mucopolysaccharides. Some sections were treated with sodium iodate as controls in place of the oxidation with periodic acid before the application of Schiff's reagent. When the slides were examined, it was found that there was strong cytoplasmic coloration in both control and test slides. It seems probable that the permanganate oxidizes some component of the cell to aldehydes, which then interfere with the PAS test; it is not therefore possible to form any conclusions as to the presence or absence of carbohydrate material by use of the PAS test after permanganate treatment.

Discussion

Several main conclusions emerge from the experiments with artificial gels.

First, treatment with permanganate causes considerable initial swelling, while treatment with osmium tetroxide leads to shrinkage. This may have some bearing on any measurements taken from electron micrographs of materials treated by these techniques.

Secondly, the rate of penetration of permanganate is slow compared with that of other histological fixatives, and the *K*- value falls off with time. The only other common fixative showing a similar behaviour is osmium tetroxide. It is significant that these two substances are the only ones so far discovered which have found widespread use in electron microscopy. The probable reason is that both substances form insoluble electron-dense reduction-products by reaction with tissue constituents, thus giving rise to image contrast by electron scattering. The fall-off in the rate of penetration noticed with these two substances may be directly correlated with the formation of some 'physical barrier' in the form of a particulate deposit in the material, as suggested by Baker (1958). The general granular appearance, so characteristic of material 'fixed' with permanganate and already noted by Luft (1956), is almost certainly due to the deposition of a rather coarse (50 to 100 Å) precipitate in the cell by the reaction of the permanganate solution remaining in the tissues with the first dehydrating alcohol.

Thirdly, none of the gels tested was stabilized in form by permanganate against the action of hot water, although osmium tetroxide treatment both preserves the form of the gel and at the same time renders it insoluble. Since permanganate does not preserve the form of proteins or of phospholipids, it cannot be regarded as a true fixative for these substances. Osmium tetroxide, on the contrary, can justifiably be regarded as a true cytological fixative.

The histochemical studies of tisues 'fixed' with permanganate confirm the

---

FIG. 6 (plate). Electron micrographs of A, a pancreas cell which has been treated with permanganate. Lettering as in fig. 5, A.

B, the endoplasmic reticulum of a permanganate-treated cell, at the same magnification as fig. 5, B. Note that there are no RNA particles adherent to the membranes and that the distance between the membrane pairs is slightly increased.

observations first reported by Luft that the DNA in the nucleus is preserved, whereas the cytoplasmic RNA is absent. Basic proteins in the nucleus are absent and must be presumed to have been leached out by the action of the permanganate solution and the subsequent dehydration and embedding processes. Phospholipoproteins, on the contrary, are 'unmasked' and the phospholipid component may then be revealed in sites which correspond to regions of high content of endoplasmic reticulum as seen in the electron microscope.

It therefore appears that although potassium permanganate is an excellent medium for revealing and studying membrane structures within the cell, it is not in fact a true fixative. The actual fixation of the tissue is performed by the subsequent treatment with alcohol.

We wish to express our sincere appreciation to Dr. R. Barer for invaluable discussions throughout this work. We also wish to acknowledge the generous loan by the Wellcome Trust of a Siemens Elmiskop I electron microscope and accessory equipment.

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The Initial Cooling of Tissues in the Freezing-drying Technique

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(*From the Moredun Research Institute, Gilmerton, Edinburgh*)

With one plate (fig. 2)

SUMMARY

When a mixture of dry ice and pentane is subjected to high vacuum for one hour the temperature of the mixture is reduced to −125° C.

The low temperature of the dry ice/pentane mixture persists for about ½ h and rises only very slowly thereafter.

The chilled dry ice mixture when used for the initial cooling of tissues produces good cytological preservation.

INTRODUCTION

IT appears to be the general consensus of opinion that initial cooling or freezing of tissues must be carried out as rapidly as possible in order to prevent great distortion of tissue structure caused by slow formation of ice crystals. The practice in recent years has been to immerse small pieces of fresh tissue in a liquid such as isopentane cooled to about −165° C by means of liquid nitrogen (Hoerr, 1936). More recently, however, Bell (1952) stressed the importance of still lower temperatures and advocated the use of propane cooled to −187° C instead of isopentane. Lacy and Davies (1959) objected to isopentane on the grounds that its mixture with liquid nitrogen is explosive and suggested the use of freon–12.

The above variations concern the cooling liquid, while the use of liquid nitrogen was accepted. In the present writer's experience, however, it is the liquid nitrogen that presents the major difficulty, especially for routine work when a number of blocks of tissue have to be removed from an animal and chilled in quick succession. Apart from the actual dangers from the use of liquid nitrogen, the temperature of the cooling mixture is very variable and rises very rapidly with the quick volatilization of the liquid gas. In order to maintain a low temperature, repeated addition of liquid nitrogen is usually required, a procedure which is very tiresome; and even so the rapid transfer of the quickly frozen pieces of tissue to the freezing-drying apparatus is still necessary in order to prevent their thawing.

In view of the difficulties involved in the routine use of liquid nitrogen mixtures a method has been evolved in which the very low but rather unstable temperature of the liquid nitrogen mixtures has been replaced by a higher temperature maintained for a much longer period. The results obtained have been compared by cooling pieces of tissue from the same organ in the following three ways: (*a*) liquid nitrogen; (*b*) mixture of isopentane and liquid nitrogen; and (*c*) CO_2/pentane mixture. Histological examination of sections proved

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that while liquid nitrogen by itself caused considerable distortion of tissue, especially in tubular organs such as kidneys, the CO_2/pentane mixture produced preservation of tissue as good as the isopentane / liquid nitrogen combination. In the large neurones of the brain, however, the distortion of the Nissl substance was less marked in the CO_2/pentane mixture than in isopentane chilled with liquid nitrogen.

PROCEDURE AND RESULTS

The effect of initial cooling and subsequent freezing-drying was studied on various tissues from 12 sheep, 3 guinea-pigs, 3 rabbits, and 3 mice. The animals were killed by complete bleeding and the organs were removed in quick succession. The following organs were usually taken: brain, spinal cord, pituitary gland, thyroid, adrenal, pancreas, kidney, liver, spleen, and lymph glands. Pieces of tissue varying in length from 2 or 3 mm to 10 or 20 mm and about 2 mm in thickness were cut with a very sharp knife from each organ immediately after its removal from the carcass, and were immersed in the cooling liquid. As a rule the whole procedure of destroying the animal, removing the required organs, cutting out about 30 pieces, and initial freezing them did not exceed 25 min.

The actual initial cooling was carried out in pentane (boiling-point 30° to 40° C), chilled to $-125°$ C by means of CO_2 / dry ice in the following apparatus. A wide-mouthed vacuum jar, 210 mm high and 90 mm in diameter, was filled with finely broken 'dry ice' (CO_2), to about 40 or 45 mm from the bottom. A cylinder of perforated zinc, 65 mm high and 40 mm in diameter, was placed on top of this, and the space between it and the wall of the vacuum jar was filled with finely broken dry ice (fig. 1). Enough pentane was then poured in to damp all the dry ice and to fill the perforated zinc cylinder. The vacuum jar with its contents was next placed in the large drying tube of the Edwards Bone-and-Artery 'Freeze-Dryer', model BA2, and subjected to high vacuum for 1 h. After the pump had been stopped and the vacuum jar removed from the drying tube, blocks of tissue placed on tinfoil were very rapidly immersed in the pentane inside the perforated zinc cylinder. The temperature of the pentane ranged between $-125°$ C and $-126°$ C and remained so for about 10 to 12 min, and thereafter rose by about 1° C every 3 or 4 min. On the average the temperature of the pentane after $\frac{1}{2}$ h (the vacuum flask being kept on the bench at room temperature of 22° C) was $-120°$ C, after 1 h $-112°$ C, and after 80 min $-108°$ C.

After the initial cooling frozen blocks were transferred to small tissue baskets partly filled with solid CO_2, and these were placed in chilled glass specimen tubes situated inside the drying tube of the Edwards BA2 at $-30°$ C for subsequent freezing-drying. It is worth noting that the Edwards BA2 was slightly modified by the fitting of an additional moisture trap mounted above the rotary pump, and of an isolation valve in the pumping line. This enabled P_2O_5 to be renewed in the additional moisture trap without breaking the vacuum. Depending on the size and number of the tissue blocks, the drying

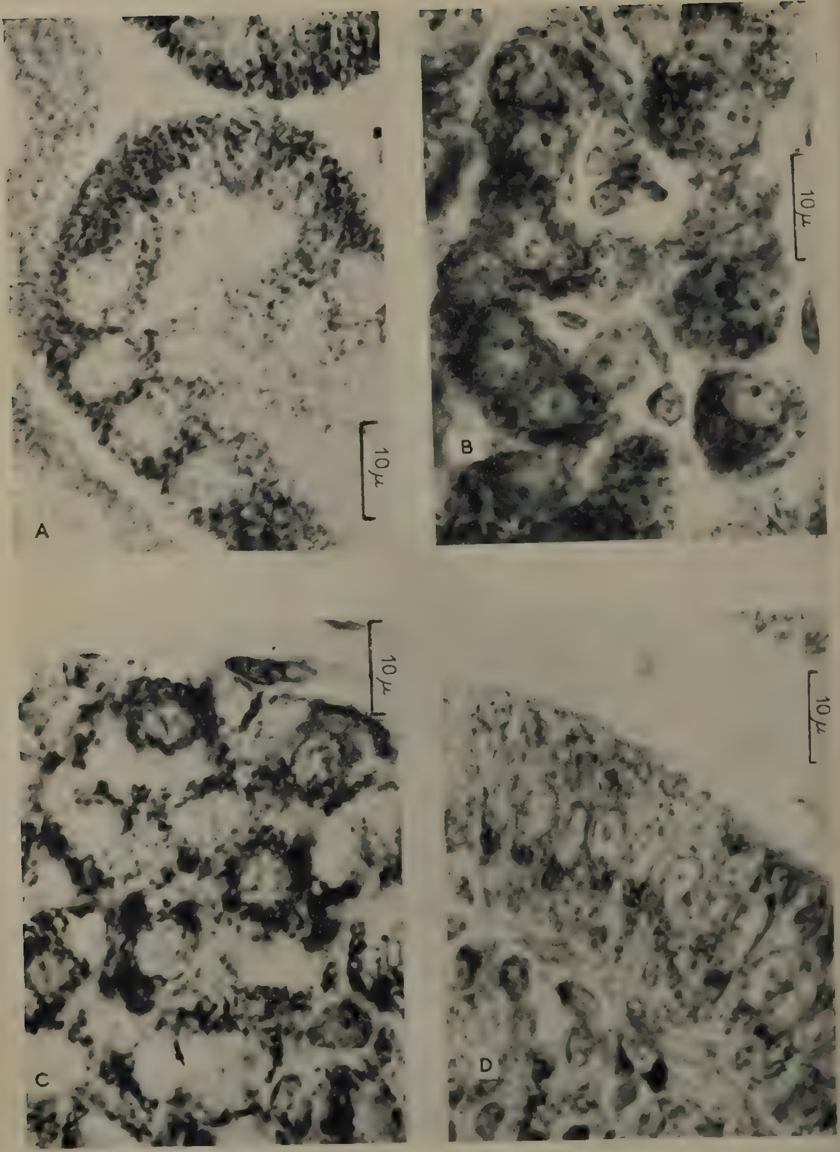

FIG. 2

I. ZLOTNIK

time varied between 48 and 72 h. After freezing-drying it has been found to be an advantage to place the thoroughly frozen-dried blocks in a desiccator at room temperature in vacuum over calcium chloride for a further 1 or 2 days. Embedding was carried out in a vacuum bath at 56° C for 4 min. Sections were cut on an ordinary rotary microtome and mounted on slides either dry for fluorescent microscopy or on 3% formalin solution in 1% calcium chloride for cytology. Various staining techniques have been used for testing the efficiency of the freezing-drying procedure, but it was found that staining for mitochondria by the method described by Chang (1956) was the most reliable.

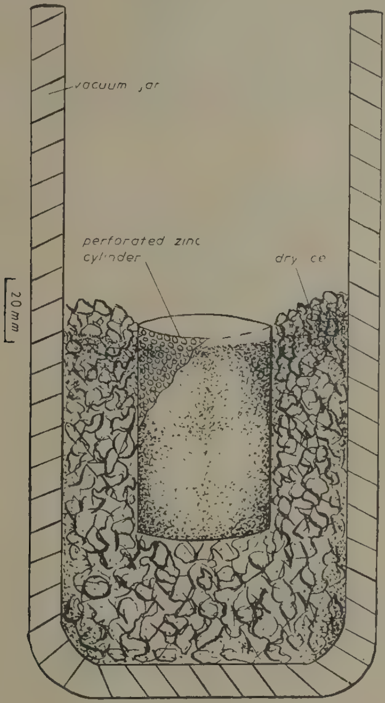

FIG. 1. Vacuum jar for initial cooling of tissues.

The results obtained with all the tissues proved very good and consistent.

FIG. 2 (plate). Sections of tissues, stained by Chang's method.

A, convoluted tubule in kidney of sheep.

B, adrenal cortex of sheep.

C, liver of guinea-pig, showing fatty changes.

D, villus of small intestine of mouse.

Good preservation of histological detail in blocks measuring up to 8 mm by 12 mm, without the appearance of zones (fig. 2, A–D), was the rule. In most organs mitochondria were easily stained and the formation of large ice crystals was not noticeable. Larger blocks of tissue showed less perfect histological preservation, especially in the centre of the section. In the central nervous system good histological pictures were obtained without any perineural shrinkage, but in the very large neurones some distortion of the Nissl substance was obvious.

The author wishes to thank Mr. J. C. Rennie for his technical assistance and also Mr. D. Watson for preparing the photomicrographs.

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Experiments on the Action of Mordants

I. 'Single-bath' Mordant Dyeing

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SUMMARY

Aluminium sulphate was chosen as the simplest mordant, and purpurine (1,2,4-hydroxyanthraquinone) as the simplest dye suitable for use in experiments with mordants.

It was shown by a series of experiments that a solution containing about 16 atoms of aluminium to each molecule of purpurine has special properties. A section already dyed with an aluminium/purpurine solution till chromatin is dark and cytoplasm lightly tinged, is in equilibrium with such a solution: that is to say, it neither takes up more dye nor loses any part of what it already holds. If the proportion of dye to mordant is increased, the section will take up more of the dye; if the proportion of the mordant to the dye is increased, the section will lose part or all of the dye it held.

The experiments described in the paper, taken in conjunction with existing knowledge of the behaviour of aluminion ions in solution and of their capacity to form chelate compounds with anthraquinonoid dyes, afford a basis for the following theory of single-bath mordant dyeing by aluminium/purpurine.

One purpurine ion chelates with one hydrated aluminium ion to form a complex ion with double positive charge; this is attracted to sites of negative charge in the tissues, such as the phosphoric groups of the nucleic acids. Electrostatic attraction is replaced by covalent bonds, resistant to solution by neutral ethanol. In extraction ('differentiation') by the mordant, the dye partitions itself between the tissue/mordant complex on one hand and the dissolved mordant on the other.

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[Quarterly Journal of Microscopical Science, Vol. 101, part 3, pp. 255–72, Sept. 1960.]

INTRODUCTION

A GREAT deal of study has been devoted to the action of mordants in the textile industry, and much knowledge of the chemical reactions involved has been obtained. The recent work on this subject, however, has nearly all been done with basic chromium salts as mordants and with azo-compounds as dyes. This combination is never used in microtechnique, where salts of aluminium and iron are the usual mordants for haematein and anthraquinonoid dyes. Beyond this, the textile worker has no easy means of discovering whether his mordant/dye complex acts as a basic or an acid dye, or indeed as neither (compare Gaunt, 1949; Race and others, 1946), while the microtechnician can generally tell by a glance down the microscope. Further, the textile worker is not concerned with the problem of differentiation (differential extraction).

A rather full account of our knowledge of the action of mordants in microtechnique has been published recently (Baker, 1958). It was emphasized that despite the important contribution of Wigglesworth (1952), large gaps in our knowledge remain.

It was thought best to concentrate at first on the simplest mordant (aluminium sulphate) and the simplest dye (purpurine) that could be used, and to try to solve one of the most straightforward problems: namely, the numerical relations between the metal atoms on one hand, and the dye molecules on the other.

If a mordant and a suitable dye are dissolved together at appropriate concentrations, and a piece of fixed tissue (section or whole mount) is placed in the solution, the tissue is dyed. If it is now placed in a solution of the mordant, the dye is seen to be extracted in clouds. The mordant solution is to some extent coloured by the dye: yet it extracts the dye. Thus one solution of mordant and dye adds colour to the tissue, while another such solution removes it. Can a balance be struck, so that a solution neither adds nor subtracts colour? And if so, what are the numerical relations between the atoms of the mordant metal and the dye molecules in such a solution?

In attempting to answer this and related questions, one is concerned with the 'single-bath' method of mordant dyeing, in which mordant and dye are dissolved in a single solution. The 'two-bath' method, involving separate solutions of mordant and dye, will be considered in subsequent papers.

In the early part of this paper it will be convenient to refer to the atoms and molecules of mordants and dyes. A consideration of the ions of these substances will be deferred to the section beginning on p. 267.

SYMBOLS FOR EXPRESSING THE CONCENTRATIONS OF SOLUTES

A lot of unnecessary words will be avoided in this series of papers by the adoption of a set of conventions for expressing the concentrations of mordants and dyes.

Since we are concerned here with numbers of atoms and molecules, solutions are made up (where appropriate) in concentrations proportionate to the molecular weight. In the case of the salts used as mordants, however, the word 'molecule' is inappropriate. The 'molecule' of potassium alum, for instance, can be written as $Al_2(SO_4)_3 . K_2SO_4 . 24H_2O$ or as $AlK(SO_4)_2 . 12H_2O$. For this reason it is necessary to select a formula for such substances and to make up solutions by reference to it. A solution containing the formula-weight in g contained in 1 l. of solution is often called an F solution. It is rather troublesome, however, to keep changing from formula-weights to molar weights as one turns from mordants to dyes. No misunderstanding will arise from the use of the symbol M throughout, if it is clear that in the case of salts, M solutions are really F solutions (the selected formula being given in every case where there could be any doubt). In this series of papers the formula with $24H_2O$ is chosen for the alums, because it shows the structure of these substances more clearly.

In the present study of mordants it was never found necessary (and it was often impossible) to dissolve any substance at more than one-tenth of the M concentration (in the sense just defined). All concentrations are therefore related to the M/10 or decimolar concentration. For brevity this will be called the dM concentration. As a general rule, mordants and dyes were used in this investigation at various concentrations in the series dM/2, dM/4, dM/8, and so on: that is to say, in the series $dM/2^1$, $dM/2^2$, $dM/2^3$. It is often convenient to express the degree of dilution in the series by mentioning the index of the denominator only. Thus 'dil. 13' means $dM/2^{13}$ (= dM/8182).

The composition of a solution containing both mordant and dye may conveniently be expressed in terms of what I shall call the *mordant quotient*: that is to say, the number of atoms of mordant metal in the solution, divided by the number of dye molecules. This figure is easily obtained from the concentrations of the two constituents expressed in terms of dM. For instance, if the mordant is at dM/4 and the dye at dM/16, the mordant quotient is $2 \times \frac{16}{4} = 8$. (It is necessary to multiply the fraction $\frac{16}{4}$ by 2, because the formula for aluminium sulphate contains two atoms of aluminium.)

Materials and Methods: General Remarks

Aluminium sulphate. Aluminium was chosen as the mordant metal for the preliminary study reported in the present paper, because its chemical reactions are simpler than those of such transition-elements as chromium and iron. The simple sulphate seemed preferable to the alum (double sulphate with potassium). Solutions were made from the crystalline salt,

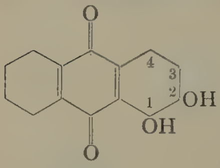
$$Al_2(SO_4)_3 . 16H_2O.$$

Crystals of analytical grade were used, but even these are not quite pure, and the actual concentrations of the solutions used must have been very slightly less than the figures suggest. The formula-weight is 630·4. In several of the experiments the salt was dissolved at dM/4. Aluminium sulphate is soluble in water at this concentration; but purpurine is much more soluble in alcoholic solutions, and 60% ethanol was therefore used as solvent. Aluminium sulphate is just soluble in 60% ethanol at dM/4 in a warm room (about 25° C).

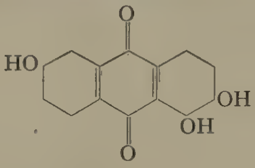
The pH of a solution of aluminium sulphate at dM/4 in 60% ethanol is 3·08.

Purpurine (molecular weight 256·2). This dye was chosen as the main subject of the investigation recorded in the present paper partly because it is simpler in chemical structure than carminic acid and haematein, and its mode of action is therefore easier to interpret; partly because it does not act as a typical basic dye in the absence of a mordant (p. 262), so that one can always be sure whether the mordant has acted or not; and partly because its solutions with aluminium sulphate, unlike those of carminic acid and especially of haematein, have scarcely any tendency to deposit an insoluble lake and thereby change in concentration.

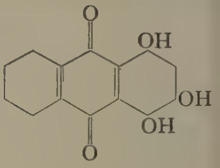
The original intention was to work with the simplest dye that is capable of being mordanted by aluminium sulphate, namely, alizarine. This, however, is not sufficiently soluble in the dye-solvents commonly used in microtechnique, and the same is true of the related anthraquinonoid dye, alizarine brown. Purpurine, another close relative, is, however, soluble in 60% ethanol, especially in the presence of aluminium sulphate. The commercial specimen of the dye was presumably not quite pure, and the actual concentrations must have been slightly less than the stated figures indicate.



alizarine



alizarine brown



purpurine

For certain purposes it was necessary to study purpurine in acid solutions in the absence of any mordant. Two such solutions were used.

Acetic purpurine (pH 3·23)

Acetic acid (glacial)	15 ml
Ethanol, 60%	up to 500 ml
Purpurine	to saturation

Sulphuric purpurine (pH 2·43)

Sulphuric acid, approx. 0·2 N	25 ml
Ethanol, 60%	up to 500 ml
Purpurine	to saturation

These solutions are yellowish orange.

The following solution was used for diluting or extracting purpurine in certain experiments.

Acid ethanol (pH 2·50)

Sulphuric acid, approx. 0·2 N	25 ml
Ethanol, 60%	up to 500 ml

It will be noted that acid ethanol has the same composition as the solvent in sulphuric purpurine.

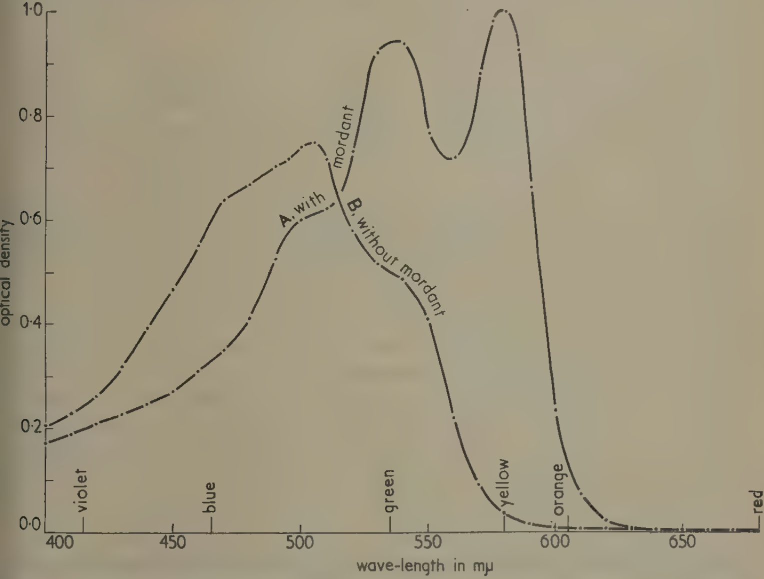


FIG. 1. Graph showing the absorption of light of various wavelengths by layers 1 cm thick of two solutions containing purpurine at the same concentration. One solution (graph *A*) was acidified by the addition of sulphuric acid; the other (graph *B*) was acidified to about the same pH by the addition of a mordant, aluminium sulphate. For full particulars see text.

Purpurine resembles carminic acid, another anthraquinonoid dye, in forming basic dye-lakes with suitable mordants. In the experiments described in the present paper it was dissolved in 60% ethanol at various concentrations from dM/16 downwards (dil. 4, 5, 6, &c.), with aluminium sulphate at various concentrations from dM/4 downwards (dil. 2, 3, 4, &c.).

These solutions are not yellowish orange, but red. The effect of aluminium on the colour of purpurine solutions is shown in fig. 1, the data for which were obtained as follows.

A measured volume of sulphuric purpurine was placed in each of two bottles, *A* and *B*. To the solution in *A* was added an equal volume of acid ethanol; to that in *B* an equal volume of aluminium sulphate at dil. 2 in 60%

ethanol. The solutions in *A* and *B* were now both strongly acid (pH 2·40 and 2·68 respectively). Both contained purpurine at exactly the same concentration; both contained sulphate ions; *B* contained aluminium ions while *A* did not. The graph shows that in the presence of aluminium ions (themselves colourless), the peak of the absorption-curve is raised and the wavelength of maximum absorption shifted from about 505 to about 580 mμ.

A solution of purpurine at dil. 4 with aluminium sulphate at dil. 2 in 60% ethanol is near the upper limit of solubility of both dye and mordant. It i convenient to call this strong solution *purpural*.

Purpural (pH 2·78)

Purpurine	1·601 g
Aluminium sulphate, $16H_2O$	15·760 g
Ethanol, 60%	up to 1 litre

Dissolve by refluxing.

Purpural may be stored indefinitely at 25° C (or equally well at 37° C), bu in a cold room there is a tendency towards the slow deposition of hydrate purpurine in the form of yellowish-orange needles. Apart from its use in th present investigation, purpural is a convenient dye for chromatin in routin histology. For such purposes it suffices to remove any precipitate by filtration but in quantitative work of the kind described in this paper it is essential t work with solutions in which no precipitate has appeared.

Aluminium sulphate at high concentrations greatly increases the solubilit of purpurine in 60% ethanol. Thus, as we have just seen, the dye can b dissolved at dil. 4 if the mordant is at dil. 2 (though the solution is on th border-line of saturation); but if the mordant is dilute, at dil. 7, the dye i near the border-line of saturation at dil. 9. In other words, the strong solutio of the mordant holds 32 times as much of the dye as the weak solution.

It would appear that purpurine tends conversely to hold aluminium sulphate in solution. Thus, if a solution similar to purpural is made up, containing only one-half of the usual weight of purpurine, there is no precipitat of dye when the solution is left in a cold room; but instead there is a slo formation of crystals of aluminium sulphate. Such crystals do not form i purpural.

The tissue chosen for study. The tissue chosen as the material on which th dye would act was the testis of the laboratory mouse, *Mus musculus*, fixed i Clarke's fluid (1851); that is to say, acetic acid mixed with 3 times its volum of absolute ethanol. (The fluid is usually ascribed to Carnoy (1886).) Th testis is convenient for work of this kind, because it possesses constituent ranging from strong basiphilia (chromosomes, and nuclei of spermatozoa) t strong acidophilia (red blood-corpuscles, and collagen of tunica propria an albuginea). Clarke's fluid contains no constituent that could act as a mordan or interfere with the uptake of a dye-lake. Paraffin sections were cut at 7 μ

In most of the experiments, dyeing continued for a week. This very lon period was chosen to ensure that equilibrium should be reached between dye solution and tissue-constituents.

Mordanted purpurine is insoluble in neutral solutions. Washing and dehydration were therefore carried out at leisure. The dyed sections were washed for 15 min in repeated changes of 60% ethanol, dehydrated, and mounted in benzene-balsam. This mounting medium was chosen because it does not extract basic dye-lakes, as xylene-balsam sometimes does. Certain unmordanted sections were dehydrated quickly, to prevent extraction of the dye (p. 262).

All observations were made with a 4-mm objective, illuminated by a $\frac{3}{4}$-cone (or thereabouts) from the condenser. When dyeing was very slight, it was necessary to use phase-contrast to find the section, but all observations of colour were made by direct microscopy, not by phase contrast. Daylight was used, or light from an electric bulb was transformed into a close approximation to daylight by passage through an Ilford 810 filter.

In the work described in the present paper, the most superficial layer of seminiferous tubules was disregarded, because its reactions to dyes were somewhat different from those of all the seminiferous tubules in the rest of the piece of tissue.

SYMBOLS FOR RECORDING THE INTENSITY OF DYEING

The standard of comparison is a section that has been dyed for 30 min in purpural, washed, dehydrated, and mounted in benzene-balsam. This has the appearance of a 'good' routine microscopical preparation, coloured by a basic dye. The chromatin is strongly dyed (red), the cytoplasm feebly.

The symbols used are as follows:

++++	More strongly coloured than the standard of comparison. The cytoplasm in particular is obviously darker.
+++	Indistinguishable or scarcely distinguishable from the standard.
++	Paler than the standard, but colour can still be seen in the cytoplasm.
+	No colour is visible in the cytoplasm, but a tinge remains in the chromatin. (The chromosomes at synizesis retain the last trace, when everything else, including even the nuclei of the spermatozoa, has lost it.)
○	No colour can be seen in the section, which can scarcely be found without the aid of phase-contrast.

These symbols are applicable to most of the experiments recorded in this paper.

The pH readings recorded in this paper were made with the Model 23A meter of Electronic Instruments Ltd. The second place of decimals is not quite reliable. The readings of optical density (fig. 1) were made with the SP 500 spectrophotometer of Unicam Instruments Ltd.

Description of the Experiments

It was necessary to do a lot of preparatory work in order to design critica experiments that would give clear answers to particular questions. To sav space, the results of this work will not be recorded here. It will suffice to sa that no results were obtained that conflicted with those given by the experiments in their final form, as described below.

The experiments are numbered for ease of reference.

Experiment in which the dye was used without mordant

Experiment 1. If a substance were capable of acting as a basic dye in th absence of a mordant, it would be unsuitable for use in a study of the kin described in this paper, because there would be doubt whether the mordan had acted as such in any particular experiment. In simple ethanolic solutions purpurine is a very weak acid dye. Certain acid dyes, however, become basi when acidified (Seki, 1933 *a*, *b*, *c*). Since solutions of aluminium sulphate ar acid, it might be thought that this salt could convert purpurine into a basi dye by acidity alone. It was therefore necessary to find out whether acidit would convert purpurine into a basic dye.

A section of the standard material was brought down to 60% ethanol an dyed for a week in acetic purpurine (pH 3·23). It was then passed directl into absolute ethanol, left in changes of this for only 1½ min, and mounted i benzene-balsam. The action was that of an extremely feeble acid dye. N special affinity for chromatin was shown. The cytoplasm of the spermatogenetic cells was a very faint brownish yellow, that of the interstitial cell slightly darker; the collagen of the tunica propria of the tubules somewha darker again.

If purpurine is made up at dil. 9 in a solution of aluminium sulphate at dil. in 60% ethanol, the pH of the fluid is 3·28; that is to say, the acidity is almos exactly the same as that of acetic purpurine. This solution, however, acts o the tissue in an entirely different way. It is a typical, bright red, basic dye with special affinity for chromatin, but none for the collagen of the tunic propria.

This experiment shows that when purpurine acts as a basic dye in th presence of aluminium sulphate, it is mordanted to the tissue. This is, indeed what one would expect, for the chemical formula for purpurine does no suggest any capacity to act as a basic dye in the absence of a mordant.

Experiment on the extraction of the dye-lake by the mordant and by acid

Experiment 2. It is a familiar fact that solutions of mordants are able to extract mordanted dyes from tissues. It is important to know whether aluminium sulphate extracts mordanted purpurine by simple acidity, or by some othe property of the dissolved salt.

Sections of the standard material on two slides were brought down to 60%

ethanol and dyed for 30 min in purpural. They were then washed for 15 min in changes of 60% ethanol. One slide was placed in aluminium sulphate solution at dil. 2 in 60% ethanol (pH 3·08), the other in sulphuric ethanol (pH 2·50). After 1 h both slides were washed as usual in 60% ethanol, dehydrated, and mounted in benzene-balsam.

The aluminium sulphate solution removed much more of the dye from the tissue than the sulphuric ethanol did, though it was less strongly acid. It is clear that aluminium sulphate does not extract mordanted purpurine simply by its acidity.

Experiments in which the mordant was used at constant concentration, the dye at various concentrations

Experiment 3. The main object of the experiments recorded in this paper was to determine whether particular mixtures of mordant and dye had the effect of increasing or decreasing the amount of dye held by the tissue. It was therefore necessary to use material that had already been coloured to a moderate degree. Sections of the chosen material were brought down to 60% ethanol, left in purpural for 30 min, and then washed for 15 min in several changes of 60% ethanol. One slide, the 'standard of comparison', was dehydrated and mounted. The grade of colouring was +++ (p. 261).

The other slides were placed in solutions all of which contained aluminium sulphate at dil. 2, while the concentration of the purpurine varied. In the strongest solution the dye was at dil. 4, so that the fluid was identical with purpural. In the other solutions the concentration of the dye was successively halved (dil. 5, 6, 7, &c.).

The uniformly coloured sections were left in these solutions for a week. They were then washed and mounted.

The results are shown in table 1. It will be noticed that when the dye was at dil. 5, no colour was added to or removed from the tissue during the week. The dyed tissue was in equilibrium with the mordant/dye solution. At higher or lower concentrations of purpurine the tissue gained or lost colour.

The mordant quotient of the solution in equilibrium with the standard of comparison will be called the *critical mordant quotient* (or *critical quotient*). Since the aluminium sulphate in this solution was at dM/4 (dil. 2) and the purpurine at dM/32 (dil. 5), the critical mordant quotient was $2 \times \frac{32}{4} = 16$. That is to say, in the solution that was in equilibrium with the standard, there were 16 atoms of aluminium to each molecule of purpurine. It will be understood that the critical mordant quotient was not determined exactly, because experiments were only made at concentrations of dye and mordant in the series dM/2, dM/4, dM/8, &c., not at intermediate concentrations, and because the amount of dye in the tissue was not measured accurately.

Experiment 4. The result of experiment 3 leads one to ask what would happen if *unstained* sections were placed in the same purpurine solutions as before. An experiment was therefore performed in exactly the same way as experiment 3, except that there was no preliminary dyeing of the sections with purpural. The sections remained for a week in the dye solutions. They were mounted and compared with the usual standard, namely a section dyed for 30 min in purpural.

The result of the experiment is shown in table 1.

TABLE 1

Results of experiments 3 and 4

Concentration of purpurine	*Experiment 3*	*Experiment 4*
dil. 4	++++	++++
dil. 5	+++	+++
dil. 6	++	++
dil. 7	++	++
dil. 8	++	++
dil. 9	+	+
dil. 10	+	+
dil. 11	+	+
dil. 12	+	○
dil. 13	○	○

For explanation of the symbols +, ++, &c., see p. 261.

It is clear that when the mordant quotient was at the critical figure, dyeing took place *up to* but not beyond the intensity of the standard of comparison (grade +++). Overdyeing could not occur in a solution of this critical composition.

Even when the dye was very dilute (dil. 11 = dM/2048 = 0·00125%), it was capable of imparting to chromatin a tinge that was just detectable. At this concentration there were in the solution 1024 atoms of aluminium to each molecule of purpurine.

Experiments in which the mordant was used at various concentrations, the dye at constant concentration

Experiment 5. It was necessary to use a low concentration of the dye, for otherwise it would not be possible to dissolve it at low concentrations of the mordant (even though the solvent was in all cases 60% ethanol). Dil. 9 was chosen. Aluminium sulphate was used at dils. 2, 3, 4, 5, 6, and 7. It could not be used at dil. 8 or lower concentrations, because the dye would not dissolve even at dil. 9 at lower concentrations of the mordant than dil. 7.

The sections were first dyed for 30 min in purpural and washed in the usual way. One section, the standard of comparison, was dehydrated and mounted; the rest were put in the solutions described in the preceding paragraph and left for a week. They were then washed, dehydrated, mounted, and compared with the standard.

The results are shown in table 2. The more dilute the mordant (provided that there was enough to dissolve the dye), the more deeply coloured the section.

Equilibrium with the standard was not exactly obtained. A solution with aluminium sulphate somewhere between dil. 5 and dil. 6 would give exact equilibrium. If the figure had been dil. 6, the critical mordanting quotient would have been 16, as before (experiment 3).

TABLE 2

The results of experiments 5 and 6

Concentration of aluminium sulphate	*Experiment 5*	*Experiment 6*
dil. 2	+	+ or ++
dil. 3	++	++
dil. 4	++	++
dil. 5	+++*	++
dil. 6	++++	+++
dil. 7	++++†	++++†

* Slightly paler than the standard of comparison.
† Cytoplasm almost as dark as the chromatin.

Experiment 6. This was performed in the same way as experiment 5, except that there was no preliminary dyeing with purpural. It will be seen from table 2 that the results were remarkably similar. In other words, the preliminary dyeing did not make much difference to the final result.

When the mordant quotient was at the critical figure of 16 (mordant at dil. 6, dye at dil. 9), the section took up dye until it exactly resembled the standard of comparison (+++).

The symbols shown on the bottom line of table 2 record the most strongly coloured sections seen in the whole of the present investigation. This is all the more remarkable, since the dye was extremely dilute (dil. 9). The fact is that in *all* the other experiments the mordant was at too high a concentration to allow maximum colour. It must be understood, however, that colouring is very slow when the dye is at dil. 9. The solution would not be suitable for practical use.

Experiments in which both mordant and dye were used at various concentrations, but in constant proportion to one another

Experiment 7. It was found in experiment 3 that a solution containing the mordant at dil. 2 and the dye at dil. 5 was in equilibrium with a section that had been dyed for 30 min in purpural. If the concentration of both mordant and dye were halved, quartered, &c., the number of atoms of aluminium to each molecule of purpurine would remain the same. Would there still be equilibrium?

Sections of the usual material were dyed for 30 min in purpural, washed, and left for a week in solutions containing aluminium sulphate and purpurine dissolved in 60% ethanol at the concentrations shown in table 3. It will be observed that in every case the mordant was in the same proportion to the dye, but a wide range of dilutions was tried. It was not possible to mak satisfactory experiments with the mordant at lower concentrations than di 10, because the dye gradually precipitated and the fluid became colourless.

The sections were washed, dehydrated, and mounted in benzene-balsam

The results recorded in table 3 show that if the mordant quotient is maintained at the same figure, dilution has no effect on the final result. Thus th critical mordanting quotient is independent of concentration. Equilibrium with the standard of comparison is maintained, provided that there are 1 atoms of aluminium to every molecule of purpurine. (Experiment 5 was nc in exact conformity with this finding, but the departure was small. Se table 2.)

TABLE 3

Results of experiments 7 and 8

Concentration of aluminium sulphate	*Concentration of purpurine*	*Experiment 7*	*Experiment 8*
dil. 2	dil. 5	+++	+++
dil. 3	dil. 6	+++	+++
dil. 4	dil. 7	+++	+++
dil. 5	dil. 8	+++	+++
dil. 6	dil. 9	+++	+++
dil. 7	dil. 10	+++	++
dil. 8	dil. 11	+++	++
dil. 9	dil. 12	+++	++
dil. 10	dil. 13	+++	++

Experiment 8. This was carried out in the same way as experiment 7 except that the initial dyeing in purpural was omitted. The results are show in table 3. It will be noticed that the omission of the initial dyeing made n difference to the result when rather strong solutions were used, but th sections were more weakly coloured by the dilute solutions. The instability o the dye in very weak solutions of the mordant may have affected the result.

REMARKS ON THE RESULTS OF THE EXPERIMENTS

The most significant of the 8 experiments recorded in this paper wer nos. 3, 5, and 7. The others help in the interpretation of these three.

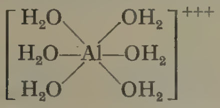
It follows from the results of experiments 3, 5, and 7 (especially 3 and 7 that a solution containing about 16 atoms of aluminium to each molecule o purpurine has special properties. A section already dyed with an aluminium purpurine solution till chromatin is dark and cytoplasm lightly tinge (grade +++), is in equilibrium with such a solution: that is to say, it neithe takes up more dye nor loses any part of what it already holds. If the proportion of dye to mordant is increased, the section will take up more of the dye if the proportion of the mordant to the dye is increased, the section will los part or all of the dye it held. An unstained section will take up dye from solution containing 16 atoms of aluminium to each molecule of purpurine until the standard degree of colouring (+++) is reached; no further colouring beyond this will occur.

It follows that a solution containing 16 atoms of aluminium to each molecule of purpurine is very easy to use in practice, since it cannot overstain; but it acts slowly. A solution that contains more than 16 atoms of aluminium to each molecule of purpurine is unsuitable for practical use, since it cannot colour sections up to grade +++.

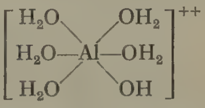
If the proportion of aluminium is reduced below the critical mordant quotient, the solution can overstain, and microscopical control is therefore necessary in practical use. If the mordant quotient is very low, there will be a strong tendency to overstain. A solution containing 8 atoms of aluminium to each molecule of purpurine is convenient. The solutes should be at high concentration in solutions intended for practical work, partly to avoid their rapid depletion by repeated use, partly to achieve fairly quick colouring of the tissues. These conditions are fulfilled by purpural (p. 260).

A Theory of Single-bath Mordant Dyeing

The experiments described in this paper, taken in conjunction with existing knowledge of the behaviour of aluminium ions in solution and of their capacity to form chelate compounds with anthraquinonoid dyes (especially alizarine), afford a reasonable basis for a general theory of single-bath mordant dyeing by aluminium/purpurine. A sketch of such a theory will be attempted here, in the belief that it will clarify thought on the subject and help in the design of future experiments. Carminic acid and haematein probably react with aluminium in much the same way as purpurine does, but both these dyes (especially haematein) have a tendency to deposit insoluble lakes when mixed with mordants.



The hydrated aluminium ion

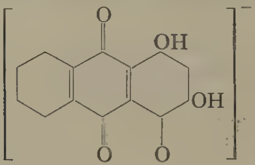


The hydrated aluminium ion after loss of a proton

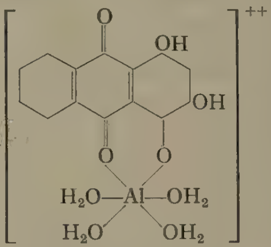
When aluminium sulphate is dissolved in water, each atom of the metal loses the 3 electrons in its outer (*M*) shell. The ion is now in a position to accept 12 electrons from suitable donor atoms. The 6 orbitals concerned are sp^3d^2 orbitals; they are hybridized, so that all are equivalent. A dative co-valency of 6 is established by the attachment of 6 water molecules through their oxygen atoms.

One (or more) of the water molecules bound to an aluminium ion loses a proton, and this accounts for the acidity of solutions of aluminium salts (Brönsted & Volqvartz, 1928). The loss of the proton reduces the charge on the ion from +++ to ++.

Other potential donors, such as alizarine or purpurine, may now replace water and thus become bound to aluminium. If the hydroxyl group in the 1-position of purpurine becomes ionized, the dye has the structure shown here. Two oxygens (the one in the 1-position, and the carbonyl oxygen nearby) are capable of replacing the oxygens of two water molecules bound to aluminium. A (soluble) chelate compound may thus be formed.



Ionized purpurine



The mordant/dye complex, according to the hypothesis here presented

Since the aluminium ion has two positive charges and the purpurine ion one negative, it might be thought that the chelate compound would bear only a single positive charge; but in fact the formation of the chelate compound is likely to be accompanied by the taking back of a proton from a hydronium ion in the solvent water, and as a result the compound ion must be supposed to possess two positive charges (Venanzi, 1959).

The reaction of aluminium sulphate with alizarine was investigated by Liechti and Suida (1885). The product was stated to be a purplish black solid, *soluble* in water and in alcohol. These authors found that in the absence of other substances, aluminium sulphate does not react with alizarine to form an insoluble lake.

It might be supposed that 3 alizarines or purpurines could replace all 6 water molecules bound to aluminium, with precipitation of an insoluble lake, and indeed this story is told in many chemical textbooks. It is true that an insoluble lake (Turkey red) is formed if calcium ions are present in addition to aluminium and alizarine; but in the compound formed by this reaction, only 2 alizarines are bound to each aluminium. Of the 2 remaining co-valencies of this metal, one still retains a water molecule, and the other is linked through oxygen with calcium (Fierz-David & Rutishauser, 1940).

A comparison of the formulae for alizarine and purpurine (p. 258) might suggest that these two dyes would react in different proportions with mordant metals. The formula for purpurine gives the impression that the hydroxyl group in the 4-position, as well as that in the 1-position, is so situated in relation to a carbonyl group as to provide means of chelation with suitable metals. There is evidence, however, that the hydroxyl group in the 4-position is unreactive, and that purpurine chelates with metals in the same proportions as alizarine (Morgan & Smith, 1922). Similarly, the hydroxyl group in the

1-position does not react with mordants unless there is another hydroxyl beside it in the 2-position (Liebermann & Kostanecki, 1887).

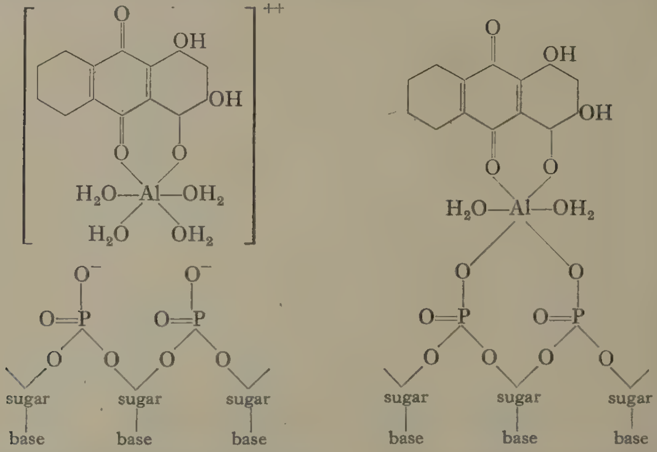
It was shown in experiment 4 (p. 263) that a solution containing 1024 atoms of aluminium to each molecule of purpurine is capable of imparting colour (though only a trace of it) to chromatin. In this case it seems certain that those few aluminium atoms that bound any purpurine at all must have bound only one molecule of it: for one aluminium can hardly be supposed to have held two purpurines, when 2047 other aluminiums held none at all. Since dyeing can take place in these and similar circumstances, the simplest hypothesis is that there is one purpurine or none to each aluminium, whether the aluminium be free in solution or attached to tissue-constituents. It is to be remembered that in all practical dyeing solutions the mordant is present in great excess.

Nevertheless, it is quite possible that when the mordant quotient is low, two dye-molecules may chelate with some of the aluminiums. If so, the resultant complex ions would carry only a single positive charge. Liechti and Suida (1885) incinerated their soluble aluminium/alizarine lake, and compared the original weight with that of the resultant aluminium oxide. Their own figures indicate that 2·69 alizarines were burnt up for each molecule of Al_2O_3 left behind. They themselves gave the figure as 2·9, and they concluded that in fact the molecule of the lake contained 3 alizarines and 2 atoms of aluminium. It is probable, however, that it consisted mostly of one alizarine chelated with one aluminium, but partly of another compound formed by the chelation of two alizarines with one aluminium.

It is thinkable that a mordant and dye, though present in the same solution, might remain separate until the mordant had attached itself to the tissue. This idea, however, is contradicted not only by the findings of Liechti and Suida, but also by independent evidence. Aluminium ions enormously increase the 'solubility' of purpurine (p. 260), and aluminium ions in solution strongly attract purpurine that has already been mordanted to the tissues (experiment 2). When the mordant is dissolved with the dye, the peak of the absorption-curve of the latter undergoes a marked heightening and shift (fig. 1, p. 259), as often happens when a chelate compound is formed.

In the presence of tissue, the positively charged aluminium/purpurine complex will be drawn towards sites of negative charge. It will accumulate in greatest abundance where these sites are most numerous. Phosphoric groups are close to one another along the whole length of the nucleic acid molecule, and the complex will therefore associate itself abundantly with chromatin. It will be attracted to protein in much smaller quantity, for the carboxyl side-groups of dicarboxylic acids will not be very plentiful even in rather strongly acidic proteins. For this reason the ground cytoplasm will be feebly dyed when the chromatin is already dark red, unless there happens to be much cytoplasmic RNA in the cell.

The results of dyeing with aluminium/purpurine show clearly that electrostatic forces determine the uptake of the mordant/dye complex, for it attaches itself to the same sites as basic dyes do. It is probable, however, that covalent linkages eventually replace the ionic bonds; for the dye is firmly held and cannot be extracted by mixtures of ethanol and water, which, as is well known, remove basic dyes from the tissues if enough time be allowed.



The mordant-dye complex attracted to a site of negative charges (a nucleic acid) in the tissue

The tissue/mordant/dye complex

Structural formulae showing hypothetically how purpurine may be linked to nucleic acid through aluminium

The hypothetical structural formula shows aluminium joined to two phosphoric groups in the same polynucleotide chain. This is not necessarily so. The two phosphoric acids might be in different chains, perhaps one in each of the two parallel chains of the DNA helix.

It will be noticed that the opposite charges of the aluminium/purpurine complex and of the two phosphoric groups will satisfy one another, and the insolubility of the product may be attributed to this. The fact that two water molecules are still attached need not confer solubility. It will be remembered that each aluminium has one water molecule attached to it even in the insoluble lake, Turkey red (p. 268).

It is not impossible that aluminium/purpurine may attach itself to some tissue-constituents by chelation. Thymine and certain other bases of the nucleic acids provide possibilities for this type of attachment. Dicarboxylic amino-acids in a polypeptide chain might also form chelate bonds with aluminium, through the —O$^-$ of the free carboxyl group and the nearest —NH—of the polypeptide backbone. Further speculation along these lines would not be profitable in the existing state of knowledge.

When a mordant/dye solution has a high mordant quotient (that is, when it contains a high ratio of aluminium to purpurine), it is probable that many aluminium ions to which no purpurine is bound are deposited on suitable sites in the tissues.

Experiments 2, 3, 5, and 7 throw light on the problem of *differentiation.* The amount of dye in a section that has been coloured by aluminium/purpurine to grade +++ (p. 261) can be reduced by exposing it *either* to an acid (for instance, dilute sulphuric acid), *or* to the mordant, *or* to any solution of mordant and dye in which there are more than 16 atoms of aluminium to each molecule of purpurine.

Acids have two possible points of attack, indicated by the oblique strokes in the expression *tissue/mordant/dye complex.* The second of the strokes indicates the easier link to break. It must be supposed that protons from the hydronium ions would tend to attach themselves to the purpurine so as to reconstitute the hydroxyl group that originally ionized and thus gave attachment to aluminium. One half of the chelate bond would thus be broken and the other thereby weakened; the purpurine molecule, unionized, would separate from the metal. In some cases (as, for instance, when acid differentiates aluminium/haematein), a striking colour change (bright blue to dull red) announces the setting free of the dye.

Sufficient acidity would also endanger the bond indicated by the first oblique stroke. Protons would reconstitute the hydroxyl groups of the phosphoric acid, thus setting free aluminium ions.

Since aluminium sulphate, on dissolving, produces protons (p. 267), it presumably acts to some extent in the same way as sulphuric or any other acid, though it would not set free any aluminium. It has been shown in experiment 2, however, that the mordant is a much stronger differentiator than an acid at the same pH, and it is therefore clear that the mordant must also act in another way when it extracts the dye. The simplest hypothesis is that the purpurine forming part of the tissue/mordant/dye complex is attracted by the hydrated aluminium ions that are still in solution, and that when these ions outnumber the purpurine molecules in the solution by more than 16 to 1, their attraction for the attached purpurine suffices to free it. The purpurine therefore partitions itself between the insoluble tissue/mordant/dye complex and the dissolved mordant/dye complex.

The metal ions must be present in great excess if they are to extract the colour from a dyed section. It thus appears that the attraction of aluminium for the dye varies according to whether the metal is free in solution as ions, or bound to tissue-constituents. Bound aluminium attracts purpurine much more strongly than its ions do.

I am much indebted to Dr. E. J. Bowen, F.R.S., Mr. F. M. Brewer, and Dr. L. M. Venanzi for giving me advice on chemical problems connected with this work. Dr. Venanzi has most generously placed his special knowledge of the chemistry of aluminium at my disposal, in repeated discussions.

Dr. Laurence Picken kindly read a draft of the paper and gave me valuable advice. Any error in what I have written must be attributed entirely to myself.

Mrs. B. M. Luke and Mrs. J. A. Spokes have both helped me a lot, in practical and clerical work respectively.

Dr. P. C. J. Brunet kindly lent me his spectrophotometer.

I take the opportunity to express my gratitude to Professor Sir Alister Hardy, F.R.S., for his long-continued support of research in cytology and microtechnique.

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The Demonstration of Glycogen in Liver Cells fixed in Osmium Tetroxide

By C. L. FOSTER

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With two plates (figs. 1 and 2)

SUMMARY

Glycogen can be demonstrated histochemically in mammalian liver cells fixed in osmium tetroxide and embedded either in gelatine or in media used in electron microscopy. The form and distribution of the glycogen correspond very closely with those of structures seen in electron micrographs and which have been presumed to be glycogen.

Evidence was obtained which suggests that the glycogen occurring in some cells as small particles is probably not associated with mitochondria.

INTRODUCTION

THE recognition of glycogen in liver cells by electron microscopy is the result of a process of elimination—the irregular areas and particles of ow density which have been identified as this material are clearly dissimilar to any of the recognized cytoplasmic organelles such as mitochondria, microsomes, &c. (Fawcett, 1955; Haguenau, 1958; Palade, 1958).

During the course of an examination of sections of rat liver prepared by Wigglesworth's (1957) method (in which the distribution of osmium in a tissue, after fixation in osmium tetroxide, is shown by subsequent treatment with progallin A), it was found possible to demonstrate glycogen in the same material by using, in the first instance, Best's carmine. The components that contain much osmium are shown in various shades ranging from black to grey, although the intensity of the coloration may be diminished by the reagents subsequently used in the demonstration of glycogen.

MATERIALS AND METHODS

Rabbit liver was the principal material used, but liver tissue of the mouse and rat was also examined. Liver specimens were obtained from normally fed animals and also from a rabbit that had fasted for 20 h and from two fed on carrots. The object of feeding on carrot for 2 or 3 days before killing was to ensure a high level of glycogen storage.

The animals were killed either by breaking the neck or by the intraperitoneal injection of nembutal. Small pieces of liver were rapidly removed and ixed for 5 h in 1% osmium tetroxide buffered at pH 7·4.

After fixation specimens of all three livers were treated with progallin A and embedded in gelatine (Wigglesworth, 1957). Sections at 5 μ were cut on

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the freezing microtome and were mounted in Farrants's medium or dehydrated, cleared, and mounted in Canada balsam. These were used to study the general cytology.

Other pieces of rabbit liver, fixed in the same way, were embedded in methacrylate or araldite. Sections of these, from 0·5 to 1·0 μ thick, were mounted on coverslips, which were then fixed to slides with Canada balsam.

Glycogen was demonstrated in the frozen sections with either Best's carmine or the PAS procedure described by Pearse (1953). Some of these sections were cut from material fixed in the same way, but with the treatment in progallin A omitted. These gave results in no way different from the others. Control sections were incubated for 2 h in diastase in tap water or saliva diluted with normal saline. The sections were dehydrated, cleared, and mounted in Canada balsam.

The sections of rabbit liver embedded for electron microscopy were prepared by the PAS method and taken through to balsam. Some of these received a preliminary treatment with diastase or saliva.

RESULTS

The general cytological features of the hepatic parenchyma in Wigglesworth preparations are shown in fig. 1, A, B. In the rabbit and mouse the mitochondria varied in shape from rod-like to spherical, but more filamentous forms were sometimes seen. Fat droplets, which colour a deep black by this method, were rather abundant in some of the cells from the rabbit's liver (fig. 1, A). Some of the slightly larger and more deeply coloured bodies resembling mitochondria proved to be lipochondria, because they were colourable with Sudan black after breaking up the osmium-polyphenol complex with weak chlorine water. Other characteristic features of the liver such as sinusoids and bile canaliculi were also quite evident. The ergastoplasmic material was not, however, demonstrable by this method.

Frozen sections of all three livers, after treatment with Best's carmine, showed the presence of glycogen in a high proportion of the cells of the parenchyma. The PAS technique gave a good overall reaction only in rabbit liver; in the mouse and the rat the reaction was confined to the outer zone of the pieces of tissue. The result with PAS always, however, corresponded very closely with that given by Best's carmine. Sections of all three livers when incubated with either diastase or saliva showed a greatly reduced or a complete absence of coloration with either method (compare fig. 1, C, D). Sections of

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FIG. 1 (plate). A, C, and D are photographs of frozen sections of rabbit liver cells prepared by Wigglesworth's method and embedded in gelatine.

B is of mouse liver cells similarly prepared. A green filter was used in photomicrography.

A shows mitochondria dark grey and fat droplets black. (Animal fed on carrots.) Farrants's medium.

B shows mitochondria. (Normally feeding animal.) Farrants's medium.

C was incubated in saline and D in diluted saliva for 2 h. Note the abundant glycogen in C in the form of large masses and smaller granules. In D the glycogen has been removed; the mitochondria, however, can still be seen. Canada balsam.

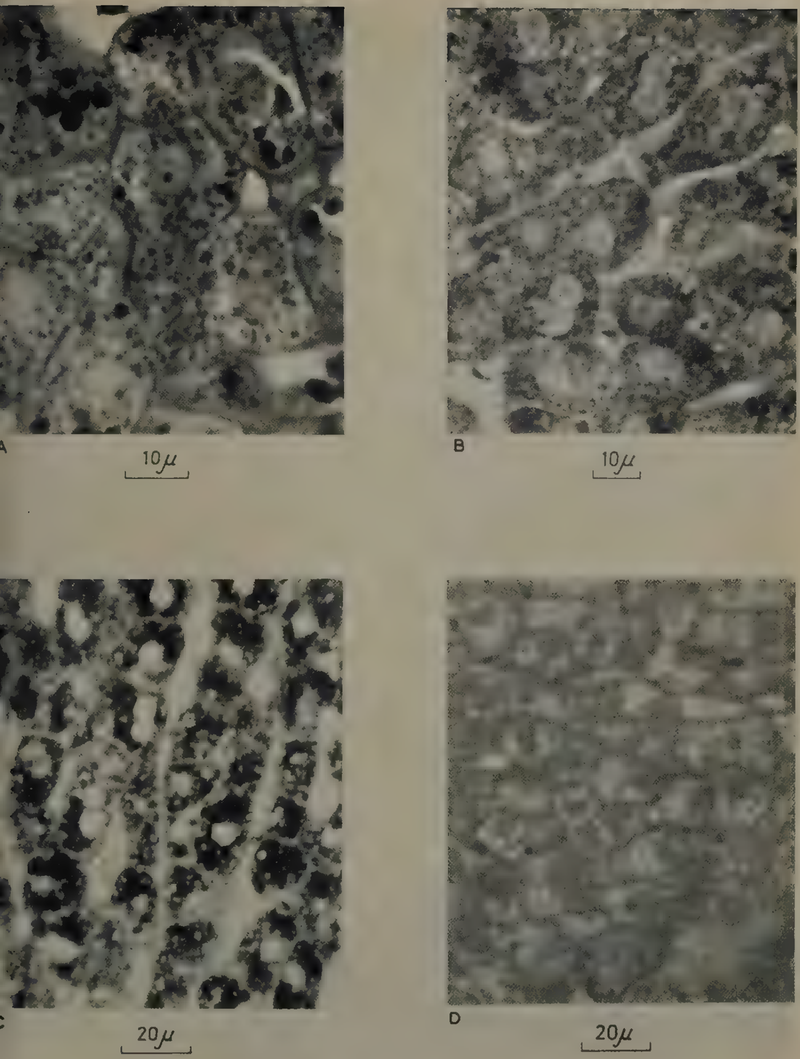

Fig. 1

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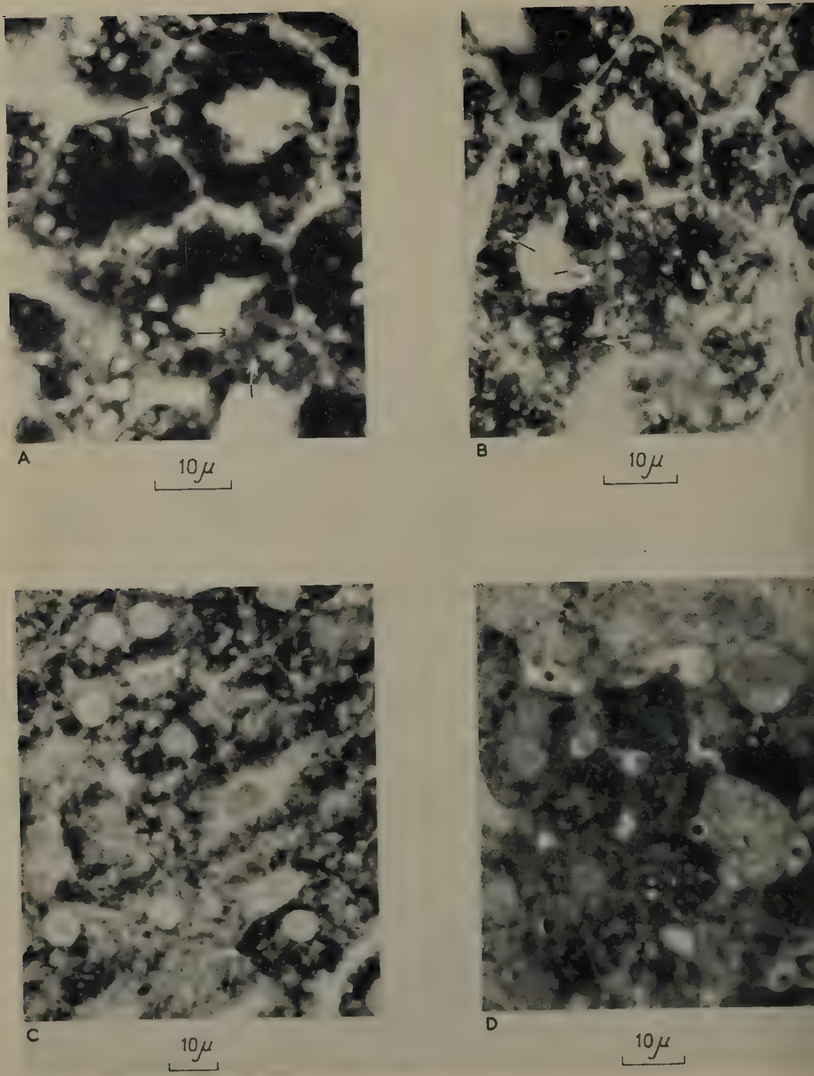

FIG. 2

C. L. FOSTER

rabbit liver which had been embedded as for electron microscopy gave positive results after the application of both staining methods, but even prolonged incubation in diastase or saliva failed to give a negative result. This was presumably due to the fact that the embedding medium prevented access of the enzymes to the sections. Nevertheless, the cytological picture (allowing for the differences in thickness of the two kinds of sections) was so similar to that seen in frozen sections that there can be little doubt that the reacting material was in fact glycogen (fig. 2, A, B).

In all livers the glycogen was evenly distributed throughout the cytoplasm. In only one instance was the 'Alcoholflucht' seen when, in some cells, the glycogen was confined to one pole. Fig. 1, C shows the distribution of glycogen in liver cells from a rabbit fed with carrot. It occurs as extensive, irregularly shaped masses and these are commonly associated with granular glycogen as well. Fig. 2, A, which illustrates more highly magnified cells, shows PAS-positive granules marked by arrows, but fig. 2, B, which is of a much thinner section of material embedded in methacrylate, shows the granular formations more clearly still, together with the large irregular masses. In these and the following illustrations the large central clear or grey areas mark the nuclei and the smaller circular clear regions (fig. 1, C, D) represent fat droplets.

Glycogen in normally feeding liver cells of the rat and mouse, demonstrated respectively with Best's carmine and PAS, is shown in fig. 2, C–D. The general pattern is similar to that seen in the rabbit—fairly large amorphous masses associated in varying degrees with glycogen in particulate form.

In this investigation particular attention was paid to the glycogen occurring in particulate form, in order to determine whether it could be related in any way to the mitochondria. In the rat, for example, much of the particulate glycogen was of about the same size as the mitochondria, but, at the same time, a substantial amount was in minutely granular form not much above the resolving limit of the microscope. The rat was particularly favourable for this kind of observation because, unlike the rabbit, neither the periodic acid nor the ammonia involved in the techniques used seriously altered the osmium-polyphenol complex in the mitochondria. As a result in some cells

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FIG. 2 (plate). All except B are of frozen sections of material embedded in gelatine. All were mounted in balsam. A green filter was used in photomicrography.

A, liver cells from a rabbit fed on carrot. Wigglesworth, PAS. These cells show dense irregular glycogen masses, and also glycogen particles in the neighbourhood of the arrows. The lipid droplets are shown as pale circular areas in the cytoplasm.

B, liver cells from a rabbit fed on carrot. Buffered OsO_4 only, PAS. Thin section of material embedded in methacrylate. The appearance is similar to A, but the thinness of the section shows the particulate glycogen more clearly. Many of the granules, particularly those near the cell boundaries, appear significantly smaller than mitochondria; others (indicated by the arrow) are of about the same size.

C, liver cells of rat. Wigglesworth, Best's carmine. In these cells the glycogen is chiefly in the form of small irregular masses. The grey mitochondria can be seen in several cells.

D, liver cells of mouse. Wigglesworth, PAS. These cells are from the edge of the tissue. The glycogen is in the form of irregular floccules.

it was possible to study the glycogen granules together with sharply defined mitochondria. This still, however, admits the possibility that those mitochondria retaining their black colour are those devoid of glycogen.

A comparison of figs. 1, A and 2, B, which illustrate rabbit liver cells, again suggests that the identification of all glycogen granules with mitochondria has little to support it, since some of the former seem to be markedly below the size range of the latter.

After treatment by the PAS method thin sections of liver (embedded in araldite) from one rabbit showed glycogen in the form of granules of uniform size evenly distributed in the cytoplasm. In this instance their resemblance to mitochondria was rather striking. When, however, similar but unstained sections mounted in Farrants's medium were examined by phase-contrast microscopy, two kinds of granule could be seen. Some were black and slightly larger, and occurred in smaller numbers than the others, which were grey and not so easily resolved. A similar examination of PAS-treated sections in Farrants's medium showed black granules distinct from the PAS-positive ones, which were smaller. From these observations it seemed reasonable to regard the grey granules as glycogen and the others as mitochondria.

Discussion

The results obtained in this study confirm the supposition that the irregular, often structureless regions of the background cytoplasm seen in electron micrographs of liver cells are in fact glycogen. Allowing for the great differences in section thickness, there does seem to be a striking correspondence between these regions and the PAS-positive or carmine-positive masses and floccules seen in the liver material described here. Considerable variation in the ultrastructure of the areas that contain glycogen has been reported and this might well be reflected in the appearances seen by light microscopy. Fawcett (1955), for example, commenting on this, states that the areas rich in glycogen '... may present a cloudy, amorphous appearance with no apparent fine structure or they may have a uniform ground-glass texture made up of many discrete 100–200 A° particles of low density. In other instances these particles are clumped to form slightly larger units which give the glycogen rich areas of cytoplasm a coarser granularity'. Thus it is possible that a larger-scale clumping could give rise to the granular glycogen encountered here.

The observations reported above, suggesting that many, at all events, of the PAS-positive granules could not be mitochondria because they occur in addition to them, is in line with the available biochemical data obtained from the analysis of subcellular fractions. This indicates that the enzyme systems concerned in glycogen formation are associated with the supernatant fluid rather than with the fractions containing the various cell organelles (Green, 1957; De Duve and Berthet, 1954; Hogeboom, Kuff, and Schneider, 1957), although after similar procedures Sacks, and others (1957) claimed a positive reaction in smears of mitochondrial and microsomal fractions.

There are reports in the literature describing the application of histochemical methods for glycogen to tissues prepared for electron microscopy (Morgan and Mowry, 1951; Bondareff, 1957), but these involve either a completely different mode of fixation or, if osmium tetroxide is used, it is only after preliminary treatment with the reagents necessary for the histochemical testing, so that osmium tetroxide acts in effect as a secondary fixative.

It has not been the purpose of this investigation to determine whether osmium tetroxide preserves glycogen at its intracellular sites more precisely than other modes of fixation (Mancini (1948) and Lison (1949), for example, assert that only freezing-drying can reveal its exact location). It has rather been to establish that what, by a process of elimination, has been designated as glycogen in electron micrographs can be shown to be such histochemically.

I should like to express my thanks to Mr. H. Long for his technical assistance and for preparing the photomicrographs.

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The Iron-containing Shell of *Gromia oviformis* (Rhizopoda)

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With one plate (fig. 2)

SUMMARY

Gromia oviformis has an organic shell or wall modified at one point to form a specialized oral region through which the protoplasm extrudes as pseudopodia. Whereas the shell appears to be predominantly proteinaceous with additional acid mucopolysaccharide and lipid components, the gel-like oral capsule, the main feature of the oral region, appears to be composed mainly of acid mucopolysaccharide.

The changes in form undergone by the oral capsule during the opening and closing of the oral region are described.

The shell, apart from the oral capsule, normally contains a large amount of ferric iron. When sections, from which the iron had been removed, were immersed in sea-water reinforced with iron or in aqueous iron solutions, it was found that iron was only taken up by the shell when the concentration was greater than 12·5 mg Fe per litre. Only when the concentration was in the region of 200 mg Fe per litre did the shell take up an amount comparable with that which is normally present. It appears unlikely that during life a reaction between the iron in normal sea-water and the shell can be wholly responsible for the iron content of the organic wall. Alternative sources of iron are discussed.

A great reduction in the amount of iron taken up from iron solutions under conditions of low pH, and after the sections have been methylated, points to the importance of carboxyl groups in the binding of iron by the shell. The nature of the union of the iron with the wall is discussed and shown to be similar in some ways to that in leather tanning processes where inorganic tanning agents are employed.

INTRODUCTION

THE detection of a large concentration of organically bound iron in the transparent and colourless shell of the rhizopod *Gromia oviformis* Dujardin has led to the study described in the present paper. The shell has previously been studied by Jepps (1926). Arnold (1952) has examined the oral region. An account of the confusion of names associated with *G. oviformis*, with comments on its systematic position, has been given by Hedley (1958).

MATERIAL AND METHODS

G. oviformis is very common in the lower levels of the inter-tidal zone around the rocky coasts of the British Isles, where it can be collected from the holdfasts of *Laminaria* sp., the under-surfaces of stones, among the fronds of some of the small seaweeds, and occasionally from the surface layer of sandy and muddy sediments. It varies in diameter up to a maximum of about 2 mm. In the present study living specimens have been used from Cullercoats, Guernsey, Jersey, Millport, and Plymouth in the British area. In addition to

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these, preserved specimens have been examined from the 1926 Discovery collections, Station 45, in the neighbourhood of South Georgia.

Much of the structural detail of the shell can be seen in unfixed, fresh, dissected material examined by ordinary and phase-contrast microscopy. Thin sections of the whole animal have been the basic preparations for the staining and chemical methods. The routine fixatives were 10% formalin, 70% ethyl alcohol, and Bouin's fluid (both cold and warmed to 60° C). After dehydration the specimens were embedded by Peterfi's celloidin-paraffin method (Pantin, 1946) and sections cut at 6 μ.

Routine staining techniques employed were Mallory's triple stain, Heidenhain's iron-haematoxylin (Pantin, 1946), and Southgate's mucicarmine (Gatenby and Beams, 1950). Chemical methods for the demonstration of certain amino-acids were the Millon reaction (Bensely and Gersh modification) for tyrosine, the Sakaguchi reaction (Baker's 1947 modification) for arginine, and the coupled tetrazonium reaction for tyrosine, tryptophane, or histidine. These methods were performed as given in Pearse (1953). The Hotchkiss (1948) procedure for the periodic acid / Schiff method (PAS), both before and after incubation with diastase, was used as a guide to carbohydrates containing *a*-glycol groups, while an indication of acid mucopolysaccharides was obtained by staining in a 0·1% aqueous solution of toluidine blue and examining in water (Pearse, 1953). The presence of lipids was shown after portions of the fresh wall had been treated with Sudan black B.

Recent accounts of the methods by which iron can be detected in sections are those of Bunting (1949), Gomori (1952), and Pearse (1953), while Wigglesworth (1952) discusses the role of iron in histological staining. The most sensitive method for ferric iron is Perls's method, which involves the production of Prussian blue when sections are immersed in a freshly prepared mixture of equal parts of 2% HCl and 2% potassium ferrocyanide. Iron that is rather loosely bound to the organic substrate is liberated and detected under these conditions, whereas it remains undetected in the absence of the acid in the mixture. It is with this type of bound iron that the present work is concerned. The other type found in animal tissues, of which the iron in haemoglobin is an example, is in the 'masked state' and very strongly bound.

STRUCTURE AND PROPERTIES OF THE TEST

Although a range of bizarre shapes of *G. oviformis* is found and has been described (Schaudinn, 1899; Jepps, 1926), the most common form is spheroidal (fig. 1, A). The animal is essentially a mass of protoplasm contained within an organic test, the wall of which is approximately 6 to 10 μ thick in an individual 1 to 2 mm in diameter. When an individual is punctured the protoplasm tends to ooze out and with the resultant decrease in internal pressure the test collapses, completely when the puncture is large. At one point in the wall there is an oral opening in the centre of a structure known as the oral capsule. Through this opening pseudopodia, or the protoplasm from which the pseudopodia originate, have access to the exterior. When pseudopodia are forming there is, as far as is known, no concomitant flow of protoplasm from the oral region back and over the test to form a surface film, or extramural layer, as there is in *Allogromia laticollaris* (Arnold, 1948). A regular flow such as this, whenever the pseudopodia develop, will tend to keep the outer surface of the test clean and free from settling bacteria and other micro-organisms, yet in *G. oviformis*, where no such film is found, the smooth surface of the test is always perfectly clean and shiny in healthy individuals, except in the oral region where mucous and associated material is invariably present. The tests of dead animals are always covered with detritus of some type and can be easily distinguished from those of living individuals.

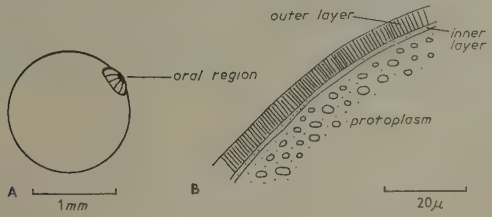

FIG. 1. A, diagram of a spheroidal *G. oviformis*. B, diagram of a section through the shell, showing the radially striated outer layer and the non-striated inner layer. The latter appears as a distinct layer in the diagram, a much simplified representation of the true nature and relationships.

The shell apart from the oral region. Previous work by Bütschli (1894), Awerinzew (1903), and Jepps (1926) has led to the conclusion that the shell is two-layered, with an outer perforated or radially striated layer and an inner structureless layer (fig. 1, B). Alternatively, the inner layer can be interpreted as an outer non-granular area of the protoplasm, a view favoured here because in preserved and stained sections the 'inner layer' is seldom attached to the outer wall and cannot be recognized as a true layer distinct from and independent of the protoplasm. Consequently the shell is considered to be basically a single-layered structure.

The radially striated nature of the (outer) shell, a constant character of both fresh and fixed preparations, has been used as a distinguishing character in classification (de Saedeleer, 1934), but it is not clear whether these striations are true perforations of the wall, or whether they reflect some regular molecular orientation within an imperforate wall. Jepps (1926) considers the structure as being 'composed of short irregularly prismatic rods of various forms and sizes each of which is perforated by one or more canals'.

From a series of sections cut tangentially through a portion of the shell there is an indication of a system of canals, each approximately 2 μ in diameter, penetrating the shell (fig. 2, E). Their constant occurrence in all sections of a series cut tangentially suggests a perforated wall. The few observations which it has been possible to make on the canal contents are consistent with the view that they are composed of a material similar to that which is found at the periphery of the protoplasm, or the supposed 'inner layer' of the shell (fig. 3).

TABLE 1

Properties of the wall and oral capsule

Method	*Outer layer (shell)*	*Inner layer*	*Oral capsule*
Polarized light	isotropic	isotropic	isotropic
X-ray diffraction	no pattern	O	O
Sakaguchi	+++	+	O
Coupled tetrazonium	+++	<+	O
PAS with diastase	++	++	++++
PAS no diastase	++	++	++++
Schiff reagent alone	O	O	O
Toluidine blue	β-metachromasia +++	γ-metachromasia <+	γ-metachromasia +++
Perls's iron	++++	O	O
Perls's without HCl	O	O	O
Sudan black B	+	O	O

From the results of various tests on sections of the whole animal (table 1) it may be deduced that the outer or true shell is entirely non-crystalline and composed mainly of a proteinaceous substance together with an acid mucopolysaccharide and some lipid material. The majority of the specimens examined have possessed shells which after Perls's method were shown to be very heavily coloured with Prussian blue. Occasionally, however, specimens have been observed in which there appeared to be rather less iron in the shell. In these cases the plugs (fig. 3) seemed to contain far more iron than the surrounding shell substance, so that the radially striated nature of the shell was very apparent. On only one occasion have animals been found which did not contain detectable iron in the shell.

In addition to the results in table 1 it is known that the shell is resistant for long periods to tap-water, to 3% acetic acid, and to concentrated HCl, both cold and boiling, whereas it is dissolved in 10 min in 50% boiling caustic potash (Jepps, 1926). It would appear from all these properties that there is no justification in continuing to use the term 'pseudochitin' proposed by Awerinzew (1907) for the cementing material of freshwater rhizopod shells and used by Jepps (1926) to describe the composition of the wall of *G. oviformis*. Vinogradov (1953) noted that the substances to which the name pseudochitin was given in the first place are proteinaceous and it is now

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FIG. 2 (plate). 6μ transverse sections through the shell, showing the outer and inner layers after treatment with A, aniline blue and orange G; B, PAS; C, Meyer's haemalum; and D, the coupled tetrazonium reaction respectively. Fixed with Bouin's fluid.

E, a portion of shell seen in tangential section, showing the perforated structure. 6μ. Fixed with Bouin's fluid and stained with toluidine blue.

F, G, longitudinal sections through the oral region, showing the relationships between the test-wall and oral capsule. 8μ. Fixed with Bouin's fluid. F, stained with Meyer's haemalum; G, with aniline blue and orange G.

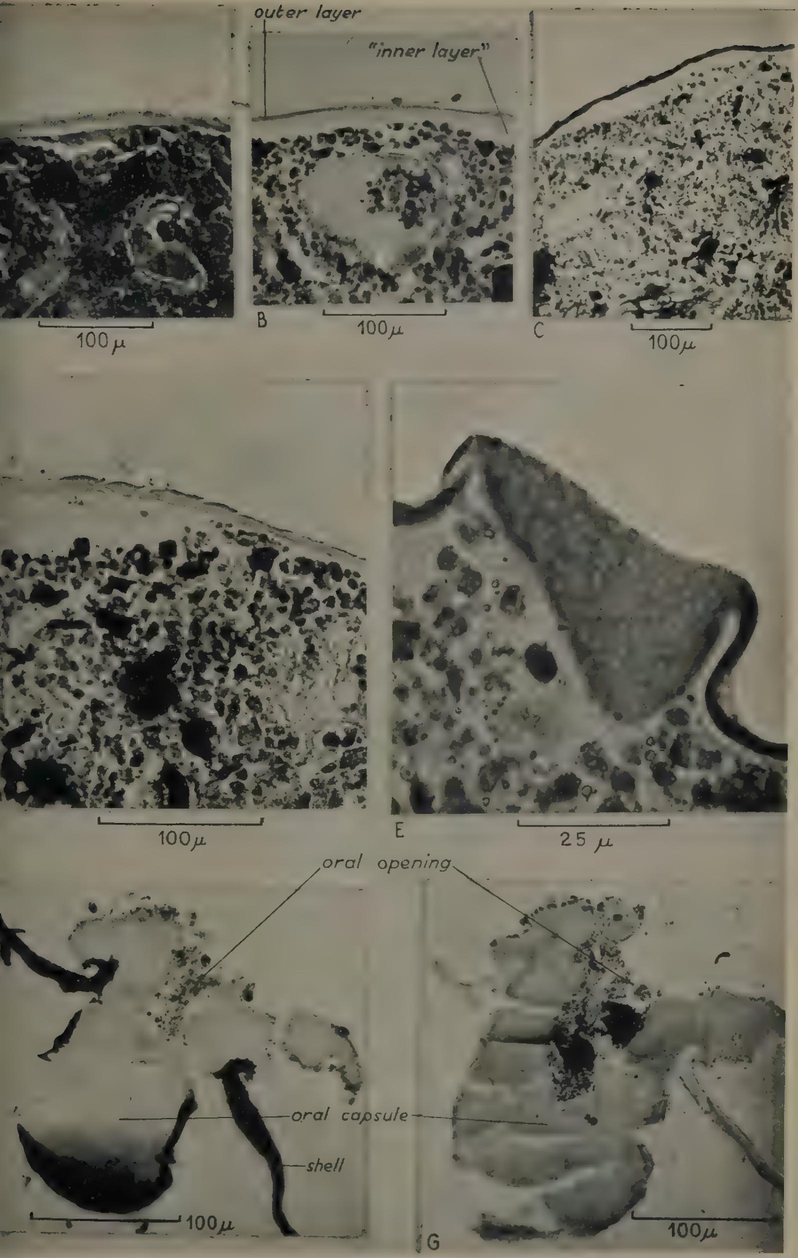

Fig. 2

R. H. HEDLEY

generally appreciated that pseudochitin bears no relation to true chitin. Examples of the formation and occurrence of chitin in the phylum Protozoa are not known (Richards, 1951; Jepps, 1956), and although X-ray diffraction studies of the cyst-membrane of the rhizopod *Pelomyxa illinoisensis* have led Sachs (1956) to conclude that it is present, there is as yet no confirmation of this.

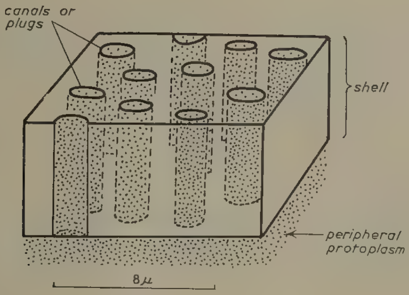

FIG. 3. Diagram of a portion of the wall to show the perforate structure. Although the plugs are represented as single independent canals, it is possible that they are interconnected.

The oral region. During and after fixation of a complete individual the oral region becomes much distorted and is invariably fixed in what will be referred to as the expanded or open position. Arnold (1952), examining fresh material, described far more of the oral structure than had been previously recognized, and his text-figure illustrates what is approximately the half-open condition. Bütschli (1894) appreciated that changes in form of the oral apparatus do take place, but it is not possible to relate his rather over-simplified diagrams of these changes to those described here. It may be noted that Bütschli was describing *G. dujardinii* M. Schultze, which is now considered to be the same as *G. oviformis* Dujardin (Rhumbler, 1903; Jepps, 1926).

When *G. oviformis* is alive and attached to the substratum the oral apparatus and the pseudopodia are protruded, and when the animal is forcibly detached, he mouth or aperture closes as the pseudopodia and protoplasm are withdrawn. It is with these movements that the following account is concerned.

In order to obtain satisfactory sectioned preparations, it is necessary to dissect the oral part of the shell from the rest of the animal and to fix it eparately. As a result of this operation the oral structure is in the closed or contracted condition and when fixed and sectioned the shell is seen to extend ound the retracted capsule (figs. 2, F, G; 4). It is difficult, therefore, to see how the supposed 'inner layer' of the wall can enlarge in the oral region to orm the 'collar', as has been suggested (Jepps, 1926), although it is possible to ee how such a conclusion could be drawn from the evidence available in ections of the whole animal alone. The transparent, gel-like, oral capsule is tained blue by aniline blue, is strongly positive to PAS, is stained pink to red by toluidine blue (table 1), and has a strong affinity for ferric iron (table 3). These properties suggest that the oral structure is composed mostly of acid mucopolysaccharide. It is difficult to say anything about the internal structure of the capsule. The bandings visible in fig. 2, G may well be fixation artifacts.

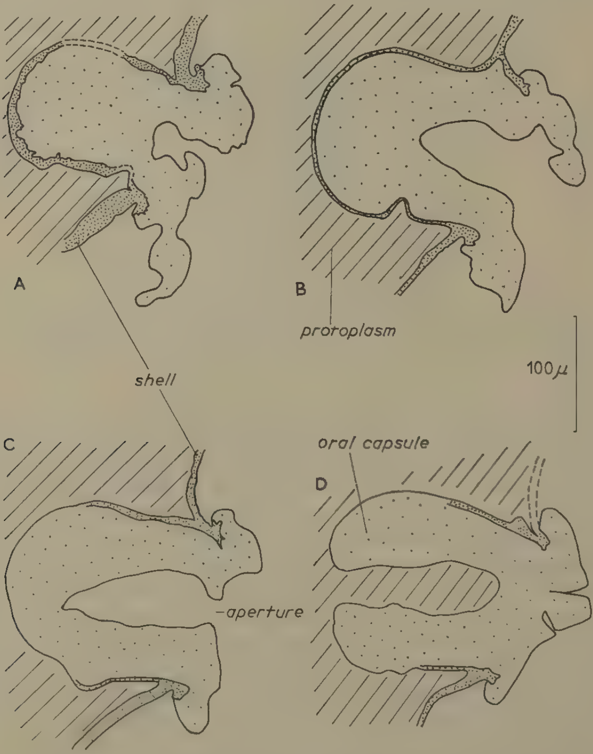

FIG. 4. Drawings of 4 sections from a series through the oral region. They show the relationships between the shell, the oral capsule and its outer layer, the protoplasm, and the test opening. (Camera lucida drawings.)

The change in form of the oral region from the open to the closed position is shown diagrammatically in fig. 5. These diagrams embody the results of examining living and preserved animals in the whole, dissected, and sectioned states. Movements and change of form imply forces capable of effecting such changes and structural components sufficiently elastic to withstand the transformation. The force is supplied by the protoplasm which pushes the oral apparatus open, and this is kept in the expanded state by the thick core of protoplasm occupying the mouth cavity (fig. 5, C). During the closing process the protoplasm withdraws inside the test and the oral region relaxes. In addition to these movements and changes in form there must also be a compensating extension of the shell near the oral region during the opening process and a relaxation during the closing movement. The perforated shell is one form of architecture which would operate in this way. The non-crystalline

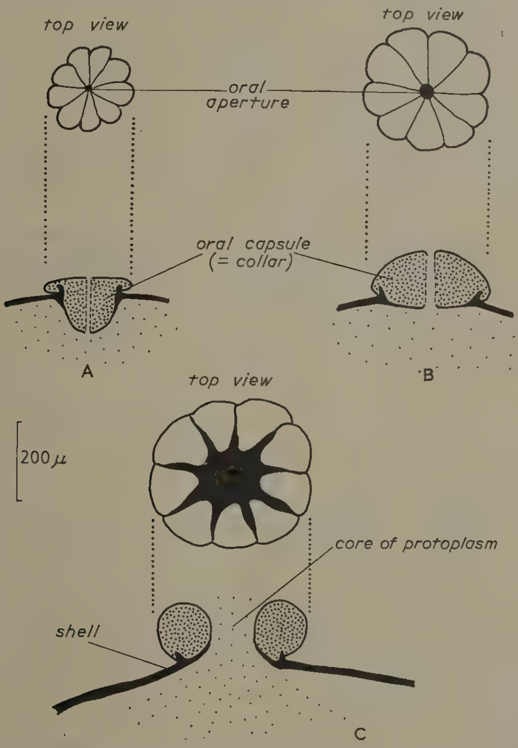

FIG. 5. Diagrams illustrating the changes in form of the oral region. A, the closed condition; B, half-open; C, fully open. From the top view this particular oral capsule is seen to be a 9-lobed structure, but the precise number is variable. The lobes are continuous with one another.

ature of the shell components as revealed by polarized light and X-ray liffraction observations may be correlated with the high degree of elasticity vhich the shell possesses.

IRON IN THE TEST WALL

It is not surprising that the ferruginous nature of the organic shell has not, the author's knowledge, been noted previously, for it is transparent and colourless, and thus offers no visual clue to the iron content. The ferric iron is detected in the wall of fixed and unfixed individuals and furthermore is preserved after fixation in Bouin's fluid. Comparisons of sections of specimens fixed in alcohol and in Bouin and treated by Perls's method failed to reveal any detectable difference in the distribution of iron in the shell or body of the animal which could be attributed to fixation in Bouin's fluid. A strong Prussian blue end-product after Perls's method is quite convincing and greater in intensity in the shell than in any other part of the animal, although the protoplasm does contain parts of algal debris and certain granules which contain iron and react positively.

Ferric iron is also present in the sheaths, tubes, or capsules of certain bacteria, sometimes collectively referred to as the 'iron bacteria' (Pringsheim, 1949 *a*, *b*; Breed and others, 1957). There are forms which secrete or cause to become deposited large iron masses such as are sometimes found in water-pipes, others with sheaths coloured brown as a result of their iron and manganese content, and others with transparent, colourless sheaths containing sufficient iron for it to be detected by the Prussian blue reaction. Similar occurrences of ferric iron are known in the colourless sheaths or envelopes of some 'iron flagellates' (Pringsheim, 1946). To this list of iron micro-organisms can be added some of the testaceous freshwater rhizopods in which iron is found in the cement which binds the inorganic plates of the test (Brown, 1950, quoted by Jepps, 1956). Likewise a number of marine arenaceous foraminifera have a ferruginous organic cement in their tests (Vinogradov, 1953; Hedley, in preparation).

Of particular interest concerning all these organisms is the source of the iron found in the various structures. It is not known whether the iron is secreted by the organisms or whether it is the result of some reaction or reactions between the iron in the medium and the substances in the shells, sheaths, or cements with which it is found associated. With this in mind an attempt was made to see if information relevant to this problem, so far as *G. oviformis* is concerned, could be obtained from some experiments with sections from which the iron had been removed. Before these are described it would perhaps be better to consider the amount of iron in sea-water and to obtain some idea of the environment in which *G. oviformis* lives.

Iron in sea-water. Owing to the low solubility of most iron compounds in alkaline medium, particularly ferric hydroxide, the amount of iron in true solution as Fe^{++}, $FeOH^{++}$, and Fe^{+++} is small, being not more than 10^{-8} mg-atom/m^3 (Cooper, 1937). Other forms in which iron may be present are classified by Cooper (1948). These include dissolved inorganic complexes, dispersed organic compounds, colloidal organic compounds, and 6 forms where the iron is present as aggregated or particulate iron such as ferric hydroxide adsorbed upon the surface of detritus and diatoms, the iron in faecal pellets, and terrigenous iron.

Although there can be no substitute for iron estimations of the sea-water actually bathing *G. oviformis* during life, some idea of the amount of iron which can be expected to be present can be obtained from published analyses. It must be borne in mind that the amount of iron in a water-mass in the sea varies both horizontally and vertically. Evidence for this is found in the papers of Cooper (1948), Lewis and Goldberg (1954), and Armstrong (1957). Higher values for iron are associated with the surface and bottom layers as compared with the rest of a water-column. In the intermediate region values up to 100 μg total Fe per litre predominate, but much smaller amounts than this are very common. The extent by which this 'basic iron content' is reinforced in the bottom layer will depend among other things on the type and nature of the bottom surface. If the amount of decaying matter is high it may be expected that the iron liberated during bacterial and other decomposition processes will result in higher total iron values in the water immediately above. This in turn will depend on the degree of mixing resulting from water movements. These, with many other possible variable factors, including the effect of the flora and fauna living in the bottom deposit, will determine the concentration of iron in the bottom water layer at any given time. It is not surprising, therefore, that analyses of this water layer in both offshore and inter-tidal areas are few. Spooner (quoted by Cooper, 1948, p. 307), commenting on total iron at different depths in the English Channel, states that 'the mean value for total iron at the bottom is larger than in the upper layers. Though the estimate for the bottom is not very precise it can be said to be roughly twice as great.'

For purposes of later comparison it is assumed here that *G. oviformis* lives in a sea-water medium containing 500 μg total Fe per litre. It will be appreciated that this is probably a very high value and that the animal will not normally be living in conditions where this concentration is exceeded.

Experiments with iron. Sections from which the paraffin wax had been removed were hydrated and placed in a 5% aqueous solution of ethylene diamine tetra-acetic acid (EDTA). This is a synthetic sequestering agent which reacts with metallic ions to form the corresponding chelate and has been used successfully with sections of *G. oviformis* for the removal of iron. Although the sequestration of ferric iron by EDTA is inhibited under alkaline conditions (Smith, 1956), the iron was removed from the sections after immersion for 4 or 5 days in the 5% solution (pH approximately 8), at least to the extent where a negative result was given after Perls's method for ferric iron.

One of the many industrial applications of synthetic sequestering agents is relevant to the present problem. These agents are used to remove metals from proteins and other materials in which the metal is present as a chelate Smith, 1956). In cases such as these, which may be similar to removing iron from the proteinaceous wall of *G. oviformis*, the synthetic sequestering agent, for example EDTA, is competing with the protein for the metal. That these compounds are effective and more efficient sequestering agents than the proteins themselves is correlated with the comparatively low stability constants of the latter (Smith, 1956).

After sections had been immersed in EDTA they were washed and placed in distilled water for a few hours. From each batch of slide-preparations treated in this way, one was tested to confirm that the iron had been removed, while the remainder were used in the iron uptake experiments. Sections from which the iron had been removed were immersed for periods of 15 min or occasionally 5 or 10 days in filtered sea-water freshly reinforced with various amounts of a solution of ferric ammonium sulphate to give concentrations of between 500 µg and 2 mg Fe per litre. Iron solutions containing 12·5 mg and 25 mg Fe per litre were prepared with glass-distilled water. After immersion in these solutions the slides bearing the sections were washed in distilled water for 5 min before testing for iron. The results obtained are given in table 2.

When colloidal ferric hydroxide or alternatively an iron salt solution is added to sea-water, the ferric hydroxide molecules tend to coalesce, flocculate, and fall to the bottom of the container (Harvey, 1937). In the case of strong iron solutions flocculation is immediate, whereas on the addition of 300 to 500 mg Fe per m^3, in sufficiently dilute solution, flocculation and sedimentation may be delayed for several days. This is noted because of the obvious danger in using old solutions. Those which have been used here have always been freshly prepared and only after standing for several days has a sediment of ferric hydroxide appeared.

TABLE 2

In all the values for total iron in the reinforced sea-water mixtures the iron content of the sea-water itself is disregarded. The amount involved is extremely small and of no significance

Period of immersion	*Vol. of sea-water* (SW) *or distilled water* (DW)	*Vol. of ferric ammonium sulphate solution* (*25 µg* Fe*/ml*)	*Conc.* Fe *per litre*	*Prussian blue*
15 min . .	49 ml SW	1 ml	500 µg	O
5 days . .	49 ml SW	1 ml	500 µg	O
15 min . .	48 ml SW	2 ml	1 mg	O
5 days . .	48 ml SW	2 ml	1 mg	O
15 min . .	46 ml SW	4 ml	2 mg	O
10 days . .	46 ml SW	4 ml.	2 mg	O
15 min . .	25 ml DW	25 ml	12·5 mg	+
15 min . .	. .	50 ml	25 mg	++
15 min . .	25 ml 0·2 N HCl	25 ml	12·5 mg	<+
15 min . .	25 ml 2 N HCl	25 ml	12·5 mg	O
Control sections without EDTA: with Perls's method . . .				++++
Control sections with EDTA: with Perls's method . . .				O

The following conclusions may be made from the results in table 2.

(*a*) The wall of *G. oviformis* does not take up enough iron from a sea-water medium containing 2 mg Fe per litre for it to be detected as Prussian blue. Iron is taken up from more concentrated iron solutions, 25 mg Fe per litre, but not to an extent which gives a Prussian blue colour-intensity equivalent o that of the normal wall. It is noted that 25 mg Fe per litre is 50 times more oncentrated than the 500 μg Fe per litre solution which is considered to be reasonable estimated amount for the areas inhabited by the animal.

(*b*) The uptake of iron and subsequent detection in the wall after immersion n a 12·5 mg Fe per litre solution is consistent with Wigglesworth's (1952) nding that iron is taken up in tissue sections from a M/4000 solution of ferric hloride. Whereas Wigglesworth noted an absorption of iron from a M/30,000 erric chloride solution, none was detected in the wall of *G. oviformis* from solution of equivalent concentration.

(*c*) The amount of iron taken up by the wall from a 12·5 mg Fe per litre olution is depressed by lowering the pH and completely prevented in a olution equivalent to N HCl strength.

It is not until sections are immersed in iron solutions containing between 12 mg and 224 mg Fe per litre that sufficient iron is taken up by the wall o give a Prussian blue positive result equivalent in intensity to that of the ormal wall (table 3).

TABLE 3

			Prussian blue reaction		
Period of immersion	*Conc. of* $FeCl_3$	*Additional procedures*	*Wall*	*Protoplasmic debris*	*Oral capsule*
5 min .	224 mg Fe/l. = M/500	O	++++	++++++	++++
5 min .	112 mg Fe/l. = M/1000	O	+++	++++	no test
ontrol sections (*a*) Perls's method only			++++	++	O
(*b*) EDTA followed by Perls's method			O	O	no test
5 min .	M/500 in acetone	methylation prior to immersion	+	+	no test
5 min .	M/500 in acetone	O	++++	++++++	no test

The following conclusions may be made from the results shown in table 3.

(*a*) The protoplasm and contents, especially the plant-debris and certain ranules, have a capacity for taking up a greater amount of iron from solution han has the wall. If a section which has not been treated with EDTA is laced in a solution of M/500 ferric chloride and then tested for iron, the hell gives a Prussian blue colour which is no more intense than in any ormal section, whereas the protoplasm takes up iron to give a much more ntense colour. This seems to suggest that the shell is normally saturated vith iron, while the protoplasm, although having a strong affinity for iron, s normally unsaturated.

(*b*) The saturation of the shell by iron, so that it resembles a normal shell n iron content, is attained only after immersion in a medium containing pproximately 200 mg Fe per litre, a concentration 400 times as great as that f sea-water considered to be rich in iron (500 μg Fe per litre).

(*c*) Methylation resulting in the blocking of carboxyl groups (Fraenkel-Conrat and Olcott, 1945) greatly reduces the amount of iron subsequently taken up. The method of esterification used here has been found by Gustavson (1954*a*) to inactivate the carboxyl groups of collagen completely, while not interfering with other protein groups.

(*d*) The oral capsule, which normally gives a negative result after Perls's method, has a strong affinity for ferric iron, which it takes up from solution.

The Nature of the Iron in the Wall

The shell is recognized as a mixed system of protein and acid mucopolysaccharide, normally containing iron which is not detected by potassium ferrocyanide or ferricyanide solutions alone in the absence of hydrochloric acid. The iron is organically bound in the sense used by histochemists, but in a different way from the very strongly bound haemoglobin iron, which requires exposure to hydrogen peroxide before it is released in a state which can be detected.

The decrease in the amount of iron taken up by the wall of *G. oviformis* when immersed in iron solutions of low pH. and also after methylation, as compared with control sections, points to the importance of the carboxyl groups in the binding of iron. At a low pH, carboxyl groups lose their negative charge and are unreactive, while after methylation the same groups are also unreactive. Other acidic groups with which iron may form non-ionizing complexes are phosphoric acid groups (Wigglesworth, 1952) and possibly sulphuric acid ester groups (Hedley, 1956). In the wall of *G. oviformis*, where there is both protein and acid mucopolysaccharide, the iron may be bound to both components through the carboxyl and sulphate groups. In the case of the oral capsule, which reacts to stains in a manner typical of acid mucopolysaccharides, the affinity for iron may be primarily due to the sulphuric acid groups present. The great reduction in iron taken up by the wall after methylation, however, suggests that most of the iron in this structure is bound through carboxyl groups. Schulman and Dogon (1954), employing a monolayer technique, demonstrated the absolute requirement of carboxyl groups in the reaction of Cr, Al, and Fe ions with amino-acids, polypeptides, and fatty acids. The importance of ionized carboxyl groups in the reaction and fixation of chromium complexes by collagen has been shown by Gustavson (1954*a*). Further, a good correlation is seen between the amount of Cr_2O_3 fixed by 10 different proteins, with the exception of keratin and silk, and the carboxylic content of the proteins (Gustavson, 1954*b*). Wigglesworth (1952), working with powdered proteins, detected an increasing intensity of iron staining after immersion in iron solution, with the increasing number of free carboxyl groups present in the different proteins.

An understanding of the union or bonds between the iron and the shell components must depend on a knowledge of their precise chemical composition, their steric properties, and the pH of medium. Wigglesworth suggests: 'In the binding of iron by protein it thus seems highly probable that the initial linkage is an electrovalent bond with free carboxyl groups. But the stability of the complexes of iron with organic compounds depends on the tendency for iron to form chelate compounds in which the ionization of the salt linkage is suppressed by the co-ordination of the iron atom with some adjacent group.'

The reaction of heavy metals with proteins is of direct importance in leather tanning chemistry. Whereas the salts of many metals can act in producing a leather, broadly defined, the best-known inorganic tanning agents are the salts of chromium, aluminium, and iron. Of these chromium is found to be the most satisfactory in so far as industrial processes are concerned (McLaughlin, 1932). The basic requirements of such a tanning process are hide (protein) and heavy metal complexes. Gustavson (1954*a*, 1956) notes that two types of chrome-collagen compounds can be formed, depending on the steric properties of the system. These are formed and characterized by having the chromium bound in one of two ways and in one of these ways the chromium complexes function as cross-linking agents. This has been noted here simply to draw attention to the fact that in structures such as the shell of *G. oviformis*, where the basic ingredients of a protein / heavy metal system normally exist, there are problems similar to those in tanning chemistry and t is not unlikely that some sort of tanning by inorganic agents does take place n certain animal structures.

THE SOURCE OF IRON

Sections of the wall from which the iron has been removed will bind an amount of iron approximately equal to that detected in normal specimens when the total iron concentration of the medium in which they are immersed s about 200 mg Fe per litre. If this is regarded as indicative of the reactivity and affinity of the normal wall it appears that the iron in normal sea-water alone is insufficient to account for the amount normally present. Further, the oral capsule has an affinity for iron at least as great as that of the wall and during life is as intimately associated with the sea-water medium, yet does not normally contain bound iron. It is suggested that scheme 1 in Fig. 6 is not wholly responsible for the iron content, although colloidal ferric hydroxide or phosphate from the medium may be adsorbed to the shell, as happens in certain diatoms (Harvey, 1937). In such instances the adsorbed colloidal or particulate iron is utilized by the diatoms after its passage through the wall (Harvey, 1937; Goldberg, 1952).

It has already been noted that the protoplasm of *G. oviformis* contains extraneous material. Sections treated with Perls's method invariably contain algal or other unidentifiable debris coloured blue and rich in iron. It is possible that as a scavenger *G. oviformis* is taking in this organic material containing bound iron, using the organic moiety as an energy source and iberating the iron in the process. This may prove to be the major source of the metal, more in fact than the animal requires for normal metabolic processes, with the result that there will normally be an excess to be excreted or stored in some way. Reference may be made here to the xanthosomes, yellow bodies 1 or 2 μ in diameter found in the protoplasm of the living animal. In dead or recently sporulated individuals they are found in the lowest part of the test, where they form an orange patch. Jepps (1926) has commented on their recognition by previous workers and has noted that they may be some form of waste product. Whether they are important in the iron balance of the animal is not known; they do, however, react with acidified ferrocyanide to give a not very strong Prussian blue colour.

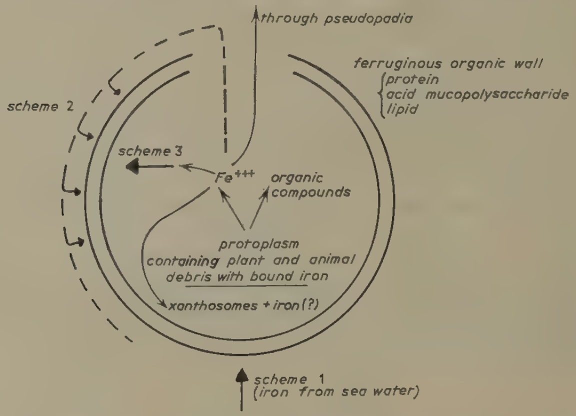

FIG. 6. Diagram of *G. oviformis* with information on the source and disposal of iron (see discussion in text).

In *G. oviformis* there is no layer of protoplasm outside the shell, such as is known in *A. laticollaris*, and therefore the possibility of scheme 2 in fig. 6 can be disregarded. This would have resulted in the wall being bathed by a fluid rich in iron.

The remaining possibilities are that the presumed excess iron is excreted or secreted by the pseudopodia and, or additionally, that the iron is deposited in the shell in some way which may be conveniently (and admittedly evasively) referred to as scheme 3 in fig. 6. This introduces a new field of inquiry which is beyond the scope of this account.

In conclusion, the results of the iron-uptake experiments indicate that the iron in the wall of *G. oviformis* is deposited or incorporated there by the activity of the animal itself. However strong this indication may be, it is appreciated that the experiments have been carried out by the use of fixed material and that there is no evidence to support an assumption that this will react in a similar way to fresh material. However, before fresh material can be used in experiments of the type described here the iron must first be removed and this will involve changes to the basic material as unacceptable as those brought about by fixation.

Part of the work described was carried out at the Marine Laboratories of Cullercoats and Plymouth, where the author has always enjoyed and appreciated the facilities and hospitality given by the Directors and staff. The author is also indebted to Dr. L. H. N. Cooper, Dr. C. H. Brown, and Dr. M. W. Jepps for their comments after kindly reading the typescript, and to Dr. R. J. Davis for undertaking the X-ray diffraction examination.

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An Electron Microscope Study of Bacteria in the Oocytes and Follicle Cells of *Blatta orientalis*

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With one plate (fig. 1)

SUMMARY

The bacteria present in the oocytes and follicle cells of *Blatta orientalis* Linn. were studied by means of electron microscopy. Bacteria are present in the follicle cells surrounding young oocytes, from where they invade the peripheral ooplasm. Each bacterium in an oocyte soon becomes surrounded by a system of membrane-like structures that form an outer covering. Later the bacteria congregate at the poles of the oocyte. An oval body was visible in one electron micrograph; this probably represents a stage in the life-history of the organism.

INTRODUCTION

SEVERAL of the earlier workers recorded the presence of 'bacteroid forms' in the oocytes of insects, including those of *Blatta* and *Periplaneta* (Wilson, 1928). In more recent years these organisms were seen by Nath and Mohan (1929) in the ooplasm of *Periplaneta americana* and by Gresson (1931) in the oocytes of *Blatta orientalis*. Bacteria present in the eggs of *P. americana* have been described and cultured by Glaser (1930). According to him the follicle cells become infected and from here the bacteria invade the oocytes where they 'form a closed layer around the periphery. Still later a definite congregation occurs at each pole' of an oocyte.

We observed bacteria in electron micrographs of young oocytes and follicle cells of *B. orientalis*. The following is a brief account of their distribution in these cells and of the outer covering that accompanies them. Little attempt is made to interpret their fine internal structure.

MATERIAL AND METHODS

The ovarioles of *B. orientalis* Linn. were speedily dissected and placed in 1% osmium tetroxide buffered with veronal to a pH of 7·4. They were embedded in a mixture of 85% *n*-butyl and 15% methyl methacrylate with 1% benzoyl peroxide as an activator. Polymerization was carried out at 60° C.

OBSERVATIONS

In electron micrographs of young oocytes of *B. orientalis* a layer of bacteria s visible in the peripheral ooplasm immediately beneath the follicle cells. The bacteria are surrounded by a system of much folded dense structures that

[Quarterly Journal of Microscopical Science, Vol. 101, part 3, pp. 295–7, Sept. 1960.]

form an outer covering extending for some distance into the surrounding cytoplasm (fig. 1, A-C). The outer covering encloses all the bacteria in a layer and in section often appears to form a network or structure resembling a honeycomb. In older oocytes a concentration of bacteria was observed at each pole (fig. 1, D).

The outer covering appears to arise in the following manner. In young oocytes each bacterium becomes surrounded, wholly or in part, by a number of thin, dense, membrane-like structures that originate from a bacterium and spread out through the cytoplasm (fig. 1, C). As these structures increase in length, those originating from neighbouring bacteria come into contact with each other and give rise to the complex system described above. It would seem that the outer coverings of adjacent bacteria do not join but become associated in such a manner as to form a continuous system enclosing all, or nearly all, the bacteria in an oocyte. As an oocyte increases in size the system of outer coverings becomes more extensive and complex in nature. The exact relationship between the outer covering and a bacterium is not always obvious. In some of our electron micrographs, however, the outer covering is clearly shown to be continuous with the outer, dense, capsule-enclosing membrane of the bacterium (fig. 1, B).

The interior of the bacterial cell is often of high density; small clear spaces are, however, frequently present (fig. 1, A, B). In some of our electron micrographs the bacteria present a different appearance. In these cases dense material is present in the form of relatively large granules lying in a material of lesser density (fig. 1, C, E).

Bacteria of varying size were shown in electron micrographs of some of the follicle cells surrounding young oocytes, and small forms, devoid of outer coverings, were sometimes visible in the peripheral ooplasm (fig. 1, C, F). The latter, we assume, are stages in the invasion of oocytes from follicle cells. This invasion appears to continue for some time.

In one of our electron micrographs an oval body is present, devoid of an outer covering but closely surrounded by a membrane composed of 4 dense components separated by clear regions. It possesses a number of dense, granular structures (fig. 1, G). This, we believe, represents some phase in the life-history of the bacteria.

FIG. 1 (plate). A, part of follicle cell and young oocyte to show bacteria in peripheral ooplasm. *fc*, follicle cell; *n*, nucleus of follicle cell; *oc*, outer covering of bacteria.

B, five bacteria in ooplasm, at higher magnification.

C, to show layer of bacteria in peripheral ooplasm of young oocyte and smaller forms devoid of outer coverings. The boundary between the follicle cell and the oocyte is poorly defined. *sf*, small forms.

D, older oocyte and part of follicle cell and its nucleus, to show concentration of bacteria at pole of oocyte.

E, bacteria in ooplasm.

F, part of young oocyte and follicle cell, to show a group of bacteria of varying sizes in follicle cell and small forms in oocyte. *sf*, small forms.

G, oval body in ooplasm. *m*, outer membrane.

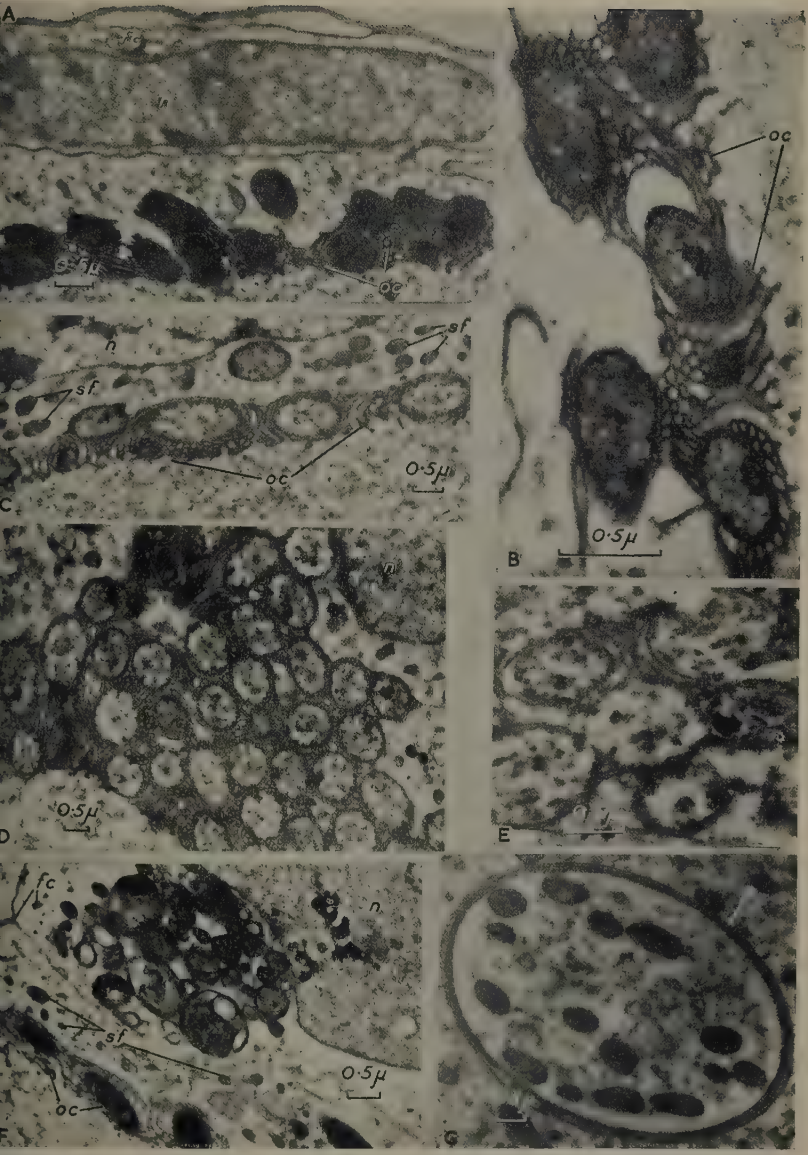

FIG. 1

R. A. R. GRESSON *and* L. T. THREADGOLD

DISCUSSION

The bacteria present in the oocytes of cockroaches, as far as we are aware, have not previously been studied with the aid of electron microscopy. In a recent contribution Nath, Gupta, and Lal (1958) conclude that the structures present in the oocytes of *P. americana* and described by other workers as bacteria are centres of lipid synthesis. They state that histochemical tests show that the bacteria 'contain phospholipid mixed with free fatty acid', and that later, when the synthesis of lipids is complete, they are negative to lipid tests. The evidence from electron microscopy shows that the bodies present at the periphery of the oocytes of *B. orientalis* are indeed bacteria, and supports Glaser's conclusions that the bacteria migrate from the follicle cells to the peripheral ooplasm, and later congregate at the poles of an oocyte.

The bacteria shown in our electron micrographs seem to possess a capsule-enclosing membrane, a capsule space, and a cell-wall similar to those recently recorded by Chapman, Hanks, and Wallace (1959) in *Mycobacterium lepraemurium*. The oval body visible in one of our photographs appears to resemble the stable L-form of *Proteus vulgaris* described by Thorsson and Weibull (1958), except that the former is surrounded by a membrane possessing 4 dense components, while the latter is enclosed by a two-layered structure.

We wish to thank Dr. Eileen O. Bartley, Department of Microbiology, The Queen's University of Belfast, for her advice during the course of this work. One of us (L. T. T.) is in receipt of a grant from the National Research Council of Canada.

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The Effects of Ultraviolet Irradiation on Uncleaved Eggs of *Xenopus laevis*

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With three plates (figs. 1, 2, and 4)

SUMMARY

The effects are described of ultraviolet (u.v.) irradiation upon the eggs of *Xenopus laevis*. The results obtained apply to fertilized eggs and also to unfertilized eggs into which blastula nuclei have been transplanted. Eggs were irradiated up to 20 min after laying, for periods varying from 15 to 50 sec.

The egg nucleus is completely inactivated by small doses of u.v. If fertilized eggs are used this gives rise to haploids, which the use of a nuclear marker has shown to be androgenetic. After irradiation the egg nucleus descends towards the centre of the egg, and comes to lie adjacent to the transplanted or sperm nucleus. At the first mitosis, however, it does not fuse but remains as a pycnotic clump in the centre of the spindle. Soon after this it disappears, without disintegrating into visible fragments. The transplanted or sperm nucleus appears to be unaffected by the irradiation and death of the egg nucleus.

The egg cytoplasm does not appear to be damaged, even after doses of u.v. which are considerably more than sufficient to kill the egg nucleus. The main reasons for this belief are that haploids obtained by other means develop no better than those obtained by u.v. An increase of u.v. treatment from 30 to 80 sec results in no increase in abnormalities sustained.

The jelly is broken down and the vitelline membrane weakened. This enables the egg to be penetrated by a micropipette without causing damage or preventing healing.

This investigation was undertaken to facilitate the analysis of nuclear transplantation experiments in *Xenopus*. The increased penetrability of eggs is of technical value for this purpose. The interpretation of these experiments is greatly facilitated by the knowledge that the egg cytoplasm need not be damaged by the u.v. and that the egg nucleus is completely inactivated so as not to interfere with the development of the egg.

INTRODUCTION

ULTRAVIOLET (u.v.) irradiation of newly laid *Xenopus* eggs has been found to kill the egg pronucleus without damaging the egg cytoplasm. This appears to be the most reliable and least damaging way of 'enucleating' the eggs of this species. The enucleation of eggs is a technique required for many different kinds of experiment; these include the enucleation of fertilized eggs for the production of androgenetic haploids, hybrid merogons, &c., and the enucleation of unfertilized eggs as in nuclear transplantation.

This paper describes the fate of the dying egg pronucleus after the u.v. treatment; this is of considerable importance in the interpretation of nuclear transplantation experiments which have been carried out with *Xenopus*

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(Fischberg, Gurdon, and Elsdale, 1958). The question whether u.v. damage has been sustained by the egg cytoplasm has been investigated.

METHODS

U.V. irradiation

The u.v. treatment of unfertilized *Xenopus* eggs has been worked out for the purposes of nuclear transplantation (Elsdale, Gurdon, and Fischberg, 1961). After the jelly has swollen by uptake of water, eggs are placed on a dry glass slide, their animal pole facing upwards; it is important to remove free water from around them since their orientation may otherwise change. The eggs are now exposed to the u.v. beam for the appropriate time (see p. 302).

The u.v. source which has been used is a Hanovia 100-W, medium pressure, mercury arc lamp; it was used without a filter but with a quartz condenser. The eggs are placed about 13 cm from the source which is mounted so as to give a downwardly directed beam.

Enucleation of the egg by microdissection

Porter's (1939) method of enucleating eggs by lifting up the egg-spot with a needle cannot be reliably applied to the eggs of *Xenopus*, since the very elastic vitelline membrane impedes the formation of an exovate. The following method has therefore been developed. It enables eggs to be enucleated with considerable accuracy (97%), but may disturb the organization of the cytoplasm. A tungsten needle is pushed through the cortex of the egg so that its shaft lies just underneath the egg-spot. The needle is then lifted tangentially away from the egg, thus stretching the vitelline membrane, which carries the egg-spot and associated cytoplasm with it. Forceps are used to pinch off an exovate from the egg, and the needle disperses the exovate into the jelly, allowing the vitelline membrane to return to the surface of the egg. The use of forceps enables the size of the exovate carrying the egg nucleus to be easily controlled. Hamilton (1957) has also obtained androgenetic haploids in *Xenopus* by a modification of Porter's technique.

Criteria for determining the success of enucleation or of pronucleus inactivation

In order to determine the efficiency of these methods, fertilized eggs were used, and a resulting haploid individual was assumed to indicate a successful enucleation or irradiation. Though a haploid nucleus is known to occasionally become diploid, this occurs very seldom and the presence of a diploid was taken to indicate a failure of the operation. Chromosome numbers were estimated in different ways. The number of nucleoli per nucleus can be counted with the phase-contrast microscope from squash preparations of embryos. Nucleoli and chromosomes can also be counted after aceto-orcein or haemalum staining of embryos or tail-tips. Because the size of nuclei varies according to developmental stages as well as ploidy, and because the haploid syndrome of abnormalities is sometimes shown by diploids, these criteria were used as an indication rather than proof of haploidy. Only those embryos which passed the blastula stage are included in the results given below.

In the case of transplant-embryos (i.e. embryos resulting from an irradiated unfertilized egg and an injected nucleus) the successful inactivation of the egg nucleus was confirmed by the use of a nuclear marker (Elsdale, Fischberg, and Smith, 1958; Fischberg and Wallace, in press). In transplantation experiments the donor nucleus possessed the nuclear marker, while the recipient egg did not. Analysis of the ploidy *and* nucleolar number of transplant-embryos shows clearly whether or not the egg pronucleus has participated in development. This only happens rarely, and when it does the transplant-embryos concerned can be excluded from the results.

Experiments and Conclusions

Evidence that u.v. irradiation kills the egg-nucleus and not the sperm nucleus

In order to investigate which pronucleus is inactivated by the u.v., experiments have been made with the *Xenopus* strain possessing a nuclear marker Elsdale, Fischberg, and Smith, 1958; Fischberg and Wallace, in press). The principle of the experiments has been to mate a normal frog, all of whose haploid) gametes are uni-nucleolate, to a heterozygous mutant frog of which half the gametes possess no nucleolus at all (0-*n*), and half are uni-nucleolate 1-*n*). The eggs were irradiated soon after fertilization, and all developed as haploids. One of two results is expected from such a cross, according to the way in which the cross is made. The haploid progeny may consist of both 0-*n and* 1-*n* individuals; the 0-*n* embryos could only have been derived from he mutant parent, while the 1-*n* embryos could have come from either parent. The gametes of a mutant parent are present in equal proportions of 0-*n* and 1-*n* (Elsdale, Fischberg, and Smith, 1958). Thus if both kinds of embryos are present in about equal numbers in the progeny of this cross, it is reasonable to assume that all the 1-*n* individuals have also come from the gametes of the mutant parent. It is only if the 0-*n*s are significantly fewer than the 1-*n*s that one must suspect that some of the 1-*n*s were derived from the non-mutant parent. The other expected result is for all the progeny of the cross to be 1-*n*s, with no 0-*n*s present at all. The absence of 0-*n*s means that none of the 0-*n* gametes from the mutant parent have escaped irradiation, but it is neverthe-ess possible that some 1-*n* gametes from the mutant parent have contributed o the progeny of the cross. The extent to which this may have occurred becomes smaller, the greater are the number of embryos in the progeny, among which no 0-*n*s were found.

In the first experiment to be carried out, a normal female was mated to a mutant male. The progeny of this cross contained 61 1-*n* haploids and 56 0-*n* haploids. These figures do not differ significantly from equality. In this experiment the 0-*n* embryos were derived from the male gametes, and there s no reason to believe that any of the 1-*n* embryos were derived from the egg pronucleus. This experiment was then repeated the other way round, by using a mutant female and normal male; the result was 117 1-*n* embryos and no 0-*n*s. This confirms the result of the previous experiment, since there is again no reason for believing that the pronucleus of any egg escaped u.v. inactivation.

Further confirmation of these results comes from nuclear transplantation experiments in which marked donor nuclei have been injected into unmarked recipient eggs. The nuclear marker shows that the great majority of the resulting embryos were derived from the injected nucleus alone. In less than 1% of cases does the egg nucleus participate in development; the reason for the failure of inactivation on these occasions is not clear, but may be due to rotation of the egg during irradiation, or to the presence of foreign matter overlying the egg nucleus.

The results of these experiments show that u.v. irradiation of the animal hemisphere of the egg selectively kills the egg pronucleus, while this is situated near the surface of the egg. Haploids which develop after such irradiation of fertilized eggs are apparently always androgenetic. The u.v. treatment in these experiments does not seem to affect either the sperm nucleus or the injected nucleus.

The effect of u.v. on the jelly and vitelline membrane of the egg

One effect of u.v. irradiation on eggs is to 'dissolve' the jelly and weaken the vitelline membrane. The latter effect of u.v. is very valuable for nuclear transplantation experiments, since the vitelline membrane cannot otherwise be penetrated by a micropipette without causing considerable damage to the egg. I wish to emphasize that this effect and its value for nuclear transplantation experiments were appreciated in collaboration with Dr. T. R. Elsdale. If too great a dose is given, the vitelline membrane fails to heal after penetration, and a large exovate is formed; too large a dose may also lead to the vitelline membrane drying on to the cortex of the egg, so causing abnormal development. With a moderate dose of u.v. the vitelline membrane heals well, but later breaks open, liberating the embryo—unharmed—a few hours before the usual hatching time. Thus it is necessary to choose an optimum u.v. dose which is sufficient to enable the egg to be penetrated without greatly distorting it, but which is not so great as to prevent healing of the injection wound. The optimum dose largely depends upon the rate at which the jelly 'dissolves' and that at which the vitelline membrane becomes penetrable. The duration of the dose, which must be determined experimentally, varies from 15 to 50 sec for the eggs of different frogs, though it is fairly constant for all eggs of any one ovulation. The reason for this variation is that eggs differ according to the ovulation in the amount of jelly they have, and in the extraneous matter attached to them. It seems to be necessary for the u.v. rays to break down the jelly before they can reach the egg nucleus, and it is probable that even in eggs which require very different optimum doses of u.v., the egg nucleus nevertheless receives the same dose.

The effect of u.v. upon the egg nucleus

Variation of the u.v. dose. The effect of u.v. upon the egg nucleus varies considerably according to the duration of the treatment (table 2), and also ccording to the length of time between fertilization and irradiation (table 1).

TABLE 1

The effect of u.v. irradiation on eggs at different intervals after fertilization

Time between fertilization and irradiation	*Total embryos analysed*	*Haploid*	*Diploid*	*Mosaics*
(min)				
5–11	31	29	2	0
12–20	53	53	0	0
21–29	23	11	11	1
30–39	23	0	23	0
40–50	18	0	18	0
Controls (no. u.v.)	187	0	187	0

TABLE 2

The effect of different doses of u.v. on the ploidy and survival of embryos derived from irradiated fertilized eggs

	Dose of U.V.				*Controls*	
	20–25 sec	*30–35 sec*	*40–50 sec*	*60–80 sec*	*Microdissection haploids*	*No treatment*
Ploidy of embryos:						
haploid	9	17	10	10	169 (97%)	0
diploid	0	0	0	0	6	34
Survival of embryos:						
total numbers	8	20	15	12	169	127
arrested late blastulae	12½%	10%	26½%	24½%	15%	0·75%
abnormal gastrulae	37½%	10%	..	..	20%	..
arrested neural folds	..	5%	6½%	..	10%	0·75%
slightly stunted post-neurulae	12½%	5%	..	8½%	20%	..
microcephalic or oedematous, (haploid syndrome)	37½%	70%	67%	67%	35%	..
normal tadpoles	..	..	..	..	..	98½%

Table 1, which includes experiments done on the eggs of 3 different frogs, shows that the nucleus of eggs irradiated within 20 min (at 20° C) of fertilization is almost always inactivated. Eggs irradiated more than 20 min after fertilization are often diploid, since the egg pronucleus has sunk far enough below the egg surface to be protected from the u.v. If the egg is not fertilized, the egg nucleus does not sink below the surface at once. Thus there is no need to irradiate unfertilized eggs for subsequent transplantation immediately after laying.

Another important respect in which the u.v. dose can be varied concerns its duration. It was mentioned above that an optimum u.v. dose is selected for each set of eggs according to their penetrability by a micropipette. It is fortunate that this same optimum dose is sufficient in nearly all cases to kill the egg nucleus, thus producing androgenetic haploids from fertilized eggs. It is of interest to find out whether this dose is the minimum capable of killing the egg nucleus or whether a reduction in it would still provide a high proportion of haploids. Table 2 shows the effect of varying the dose above and below the optimum, which in this case was 50 sec.

Table 2 and fig. 5 show that the survival of haploids from eggs irradiated for 30 to 80 sec is very similar, but that many more abnormalities are found among eggs irradiated for 20 to 25 sec. This almost certainly means that 20 to 25 sec of u.v. only partially inactivates the egg nucleus, which can then interfere with development. Embryos in the 20 to 25 sec group, though having only one nucleolus per nucleus, may well have been hyperhaploid, possessing extra chromosomes or chromosome fragments. The fact that the proportion of abnormalities found among the 3 groups of eggs irradiated for between 30 and 80 sec is the same, indicates that 30 sec or more of u.v. completely inactivates the egg nucleus, which does not then affect development. A similar effect was observed by Hertwig (1911) after X-ray treatment of amphibian eggs. Duryee (1949) has X-irradiated amphibian oocytes.

The observation that true haploids are obtained after little more than half the optimum u.v. dose, shows that this dose is more than sufficient for inactivation of the egg nucleus. It is, therefore, very unlikely that any chromosome fragments of the egg nucleus will escape complete inactivation, and then participate in development with the injected or sperm nucleus.

The fate of the irradiated egg nucleus. This investigation is based upon the analysis of 30 irradiated unfertilized eggs into which blastula nuclei were transplanted, and in which the transplanted nucleus appeared cytologically to be developing normally. The majority of these eggs were fixed during the first mitosis of the transplanted nucleus (50 to 80 min old), and the rest were

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FIG. 1 (plate). An arrow (→) indicates the egg pronucleus.

A, egg fixed 12 min after transplantation. The egg pronucleus has completed the second meiotic division.

B, egg fixed 23 min after transplantation, showing the pronucleus moving towards the centre of the egg.

C, egg fixed 25 min after transplantation, showing the egg pronucleus.

D, egg fixed 30 min after transplantation. The pronucleus is still morphologically normal.

E, egg fixed 40 min after transplantation. The dying pronucleus has a crinkled nuclear membrane and granular contents.

F, the next section of the same egg as in E, showing the transplanted nucleus (*n*) and donor cell yolk platelets (*dyp*). Part of the dying pronucleus (shown in E above) can be seen adjacent to the transplanted nucleus.

G, another egg fixed 40 min after transplantation, showing dying pronucleus. Part of the transplanted nucleus can just be seen in this section below the pronucleus.

H, the next section of the same egg as in G, showing the transplanted nucleus (*n*) and the dying pronucleus. The transplanted nucleus has a smooth nuclear membrane and a more homogeneous nucleoplasm.

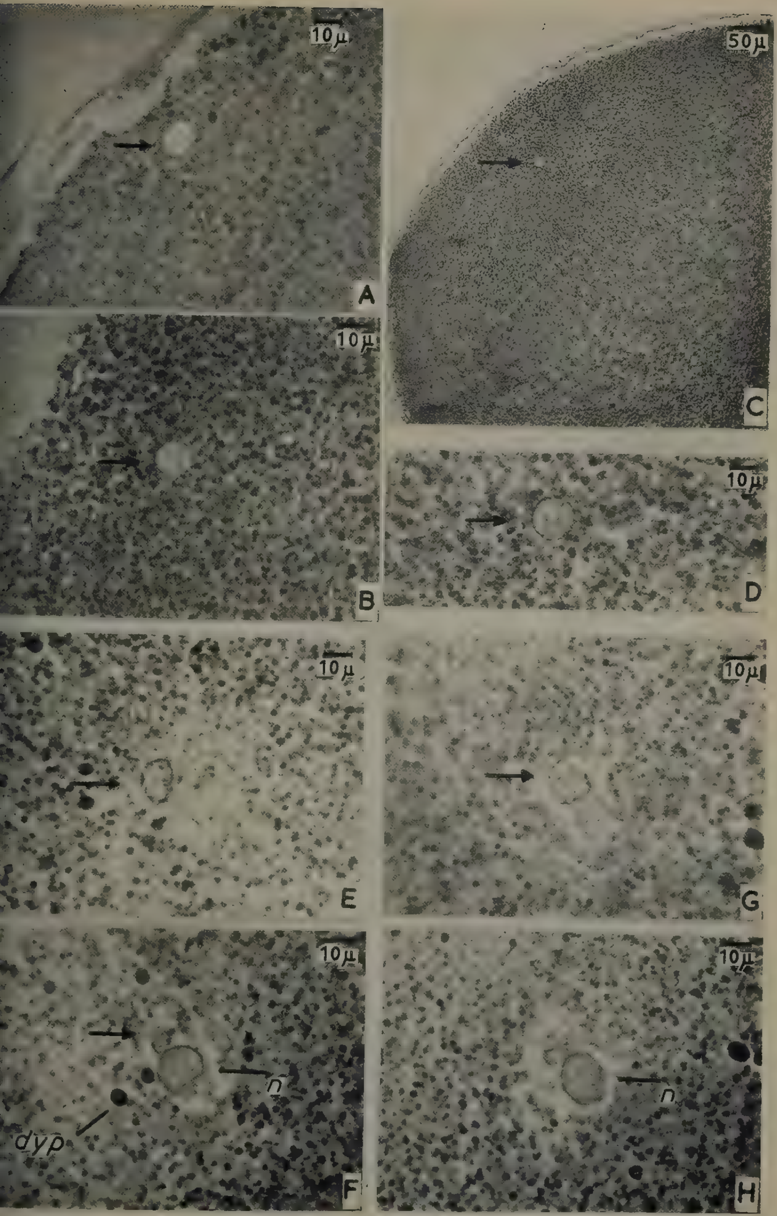

Fig. 1

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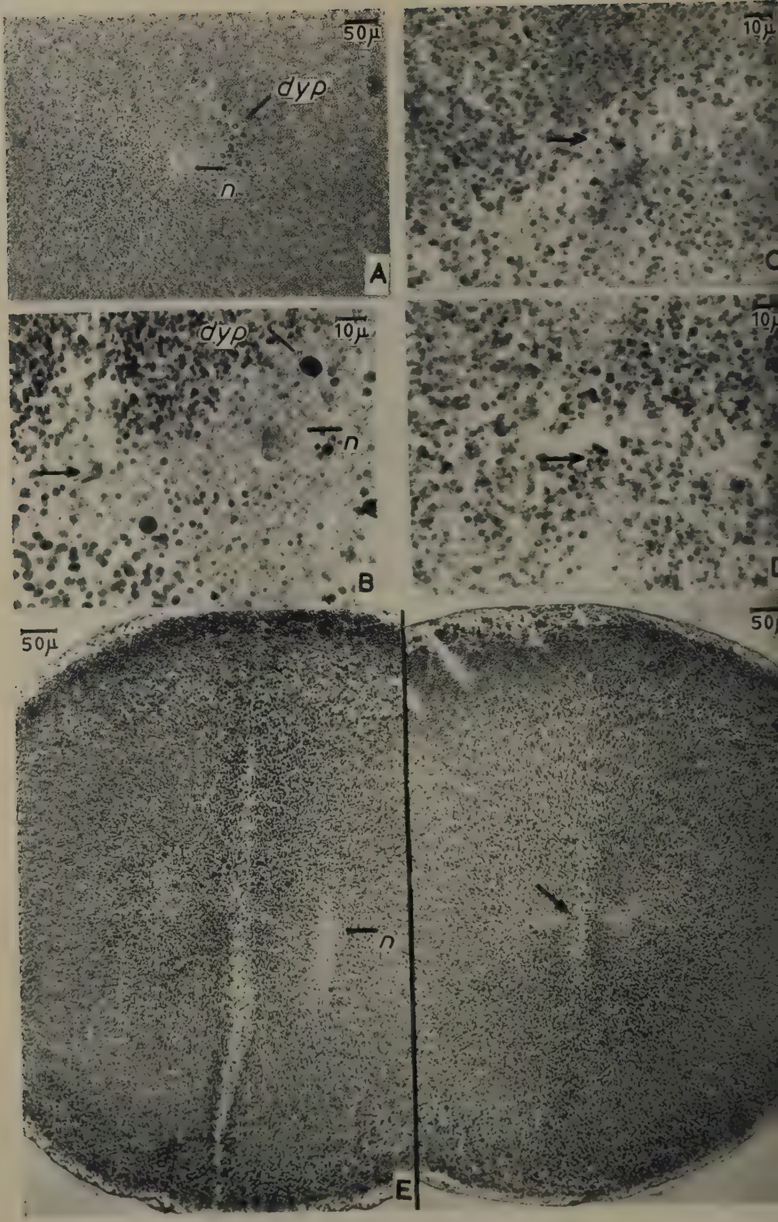

FIG. 2

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xed at various times between 5 and 50 min after transplantation. As eggs ere fixed at intervals between transplantation and the two-cell stage, it is in ost cases not possible to tell whether any one egg would have developed at ll, if it had not been fixed. In the main series of eggs with transplanted nuclei sed for this analysis, 16 of the 20 eggs fixed appeared to have normally eveloping transplanted nuclei. The remaining 4 eggs had abnormal translanted nuclei and would probably have cleaved abnormally. Of 28 unfixed ontrol transplantations done at the same time, 6 did not cleave, 4 cleaved bnormally and the remaining 18 cleaved normally and became normal adpoles. This means that the conclusions reached should be typical for eggs ith a normally developing transplanted nucleus and irradiated egg nucleus.

Eggs were fixed in Perenyi's fluid for about 6 h, and washed out overnight 1 70% alcohol. Before fixation most of the jelly was removed, and after xation the vitelline membrane was removed from a small part of the vegetal ole, to allow free passage of fluids into the egg. As the egg is very fragile when xed, it is desirable to leave the vitelline membrane to protect the rest of the gg. Serial sections at 10 μ were cut parallel to the equator; they were then tained with Mayer's haemalum, and sometimes restained with Feulgen.

When analysing these sections the position of the nuclei distinguishes the eveloping nucleus from the egg pronucleus. However, the following criteria ave been used to confirm their identification:

(1) The egg pronucleus is never surrounded by a clear yolk-free area of ytoplasm, which is always associated with the transplanted nucleus (compare g. 1, A–D with fig. 1, F, H; in fig. 1, E, G the egg nucleus is inside the clear rea associated with the developing nucleus).

(2) Nuclei of cells from the vegetal pole of the donor embryo have been sed for transplantation; these nuclei have very large (vegetal) yolk platelets ssociated with them until their first mitosis after transplantation. These large olk platelets are very conspicuous among the much smaller platelets of that art of the egg in which the transplanted nucleus lies (*dyp* in figs. 1, F and , A, B).

The events which take place after the successful irradiation of the egg

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FIG. 2 (plate). An arrow (→) indicates the egg pronucleus.

A, section showing donor cell yolk platelets (*dyp*) and yolk free region of cytoplasm around ie developing transplanted nucleus. This is the same section as is shown in fig. 1, H.

B, egg fixed 59 min after transplantation, showing the pycnotic pronucleus and a daughter ansplant nucleus (*n*); the latter, which is reforming after the first mitotic division, consists of nall spheres at this stage.

C, egg fixed 61 min after transplantation. The pycnotic pronucleus is seen in the centre of ie future cleavage furrow.

D, another egg, fixed 58 min after transplantation, again showing the pycnotic pronucleus ft between the two daughter transplant nuclei (not visible in this section).

E, egg fixed 76 min after transplantation. One of the daughter transplant nuclei can be seen), having completed the first mitosis. The egg pronucleus has disappeared by this stage.

F, egg 58 min old; this is the same section as is shown in D, containing the degenerated ronucleus. The transplanted nucleus is at anaphase of its first mitotic division (not visible in is section).

nucleus are shown in figs, 1 and 2. At the time of irradiation the egg nucleus is at metaphase of the second maturation division; in spite of irradiation it completes that division, extruding the second polar body and forming an apparently normal pronucleus. The extrusion of the second polar body has only been observed in a single egg, and it is probable that in some irradiated eggs, the whole metaphase plate sinks into the egg as a diploid egg nucleus which then degenerates. The pronucleus now moves down towards the centre of the egg (fig. 1, A–D), just as it would if it had not been irradiated. 30 min after transplantation the irradiated pronucleus is still cytologically normal. After 40 min the transplanted nucleus has reached the position where pronuclei normally fuse—about one-third of the way from the animal pole to the vegetal pole. The pronucleus joins the transplanted nucleus; by this time it has begun to degenerate, as is apparent from its crinkling nuclear membrane and granular nucleoplasm as opposed to the smooth membrane and more homogeneous nucleoplasm of the transplanted nucleus. (In fig. 1, compare E with F, and G with H. E and F, and G and H show an almost identical situation in two different transplant-embryos.) The egg pronucleus, which remains adjacent to the transplanted nucleus but does not fuse with it, now becomes rapidly smaller and more condensed. About 60 min after injection the transplanted nucleus is at anaphase of its first mitosis. The pycnotic egg nucleus is now left between the two newly formed daughter nuclei in the equatorial plane of the spindle, which is also the plane of the future cleavage furrow (fig. 2, B, C, D, F). In fig. 2, B both the degenerating pronucleus and one of the daughter nuclei of the transplanted nucleus can be seen in the same section. When these sections were restained with Feulgen, no Feulgen-positive material could be seen in the cytoplasm, even in the precincts of the strongly staining pycnotic egg nucleus. This indicates that the egg nucleus does not give rise to chromosome fragments which then become incorporated into the transplanted nucleus. 70 to 80 min after transplantation, the daughter nuclei have reached the interphase stage (fig. 2, E); in 7 embryos of this stage, no trace could be seen of the egg nucleus, which must by now have completely disappeared.

A few fertilized eggs were irradiated and fixed at various times between fertilization and the 2-cell stage. Sections of these eggs showed that the irradiated egg nucleus behaves in the same way as in eggs with transplanted nuclei. The sequence of events taking place in non-irradiated fertilized eggs and in transplant-eggs with irradiated nuclei is shown diagrammatically in fig. 3.

The behaviour of the irradiated egg nucleus described above is very similar to what is already known to apply to the nuclei of tissues that have been heavily irradiated with X-rays or u.v. (Giese, 1950; Hollaender, 1955). The susceptibility of purines and pyrimidines to destruction by u.v. can account for the finding that the egg nucleus is killed long before the egg cytoplasm is damaged. Selman (1958) has described the effect of u.v. on fertilized newts' eggs (*Triturus palmatus*). He found that the same dose of u.v. could cause ncomplete inactivation of the egg nucleus as well as damaging the cytoplasm. The reason for this rather marked difference in the results with *Xenopus* nd *Triturus* is not clear, but might be connected with polyspermy.

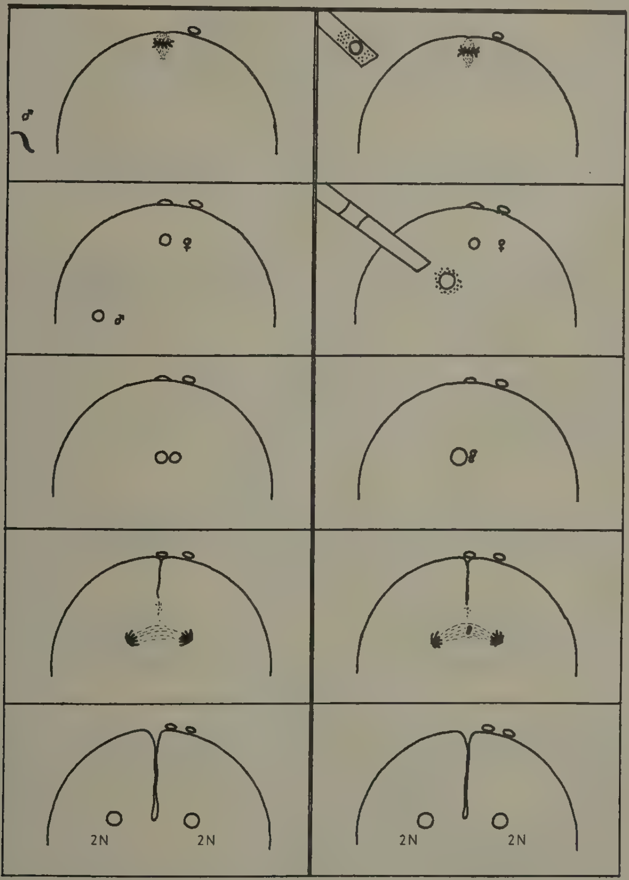

FIG. 3. Diagram comparing the development of normal fertilized eggs with irradiated eggs after nuclear transplantation. Left, normal fertilized egg. Right, u.v. irradiated egg with transplanted nucleus.

he effects of u.v. irradiation on the egg cytoplasm

The optimum dose of u.v. irradiation does not seem to cause any damage the egg cytoplasm. This cannot, however, be demonstrated clearly by any ngle experiment, so several different ones are described which all support the me conclusion.

The survival of androgenetic haploids. The extent of u.v. damage can b estimated from the number of abnormalities among fertilized eggs afte irradiation. However, eggs irradiated soon after fertilization develop a haploids, and it is difficult to be certain whether the irradiation has caused an abnormalities, since haploids are inherently abnormal owing to their reduce chromosome number. Table 2 and figs 5 and 6 show the frequency of abnormalities which have been found among embryos derived from u.v.-treate eggs, and also among haploids obtained by the microdissection metho described on p. 300. In no case did an embryo differentiate further than th microcephalic and oedematous condition referred to as the haploid syndrom (fig. 4). This is generally true of *Xenopus* haploids obtained by other mean (Rostand, 1951). Table 2 and Fig. 6 show that 70% of all u.v. haploids irradiated for between 30 and 80 sec reached the typical haploid syndrome. Sinc these embryos differentiated as far as is usually possible for haploids, this show that 70% of eggs irradiated with the optimum u.v. dose do not suffer an cytoplasmic damage.

Figs. 5 and 6 show that the remaining 30% of u.v. haploids develope abnormally. These abnormalities may be due to cytoplasmic u.v. damage, o to considerable variation in the way in which haploids differentiate. Variatio in the severity of haploid abnormalities would be expected in view of the fac that the haploid syndrome may be affected by exposed lethal genes (Subtelny 1958). Owing to genetic variation these would be present in more letha combinations in one spermatozoon than in another. Variation in the differentiation shown by haploids might also be expected to arise from poor quality eggs Transplantation experiments (Gurdon, 1960) have shown that the qualit of eggs varies considerably and poor eggs may result in abnormalities o development.

These considerations do not prove that u.v. has no harmful effect on th egg cytoplasm, but they constitute reasons for attributing abnormalities o development in fertilized irradiated eggs to other causes.

There is a tendency for eggs irradiated within 5 min of fertilization to suffe from more abnormalities of development than those irradiated after 5 mi from fertilization. These abnormalities may be due to the chance irradiatio of sperm nuclei or to a greater susceptibility of eggs to u.v. at this time. However, this source of abnormality is not thought to affect unfertilized eggs use for transplantation, since these are usually irradiated at about 20 min afte laying. By this time they have undergone a kind of 'activation', which is als observed in fertilized eggs. This is recognized by rotation of the egg, and by contraction of animal pole pigment; the egg cytoplasm also appears to becom more solid in consistency.

The effect of increasing the u.v. dose. Table 2 shows that there is no increas

FIG. 4 (plate). Photograph of androgenetic u.v. haploids (left) and diploid controls of sam age (right). The haploids were irradiated for 40 to 50 sec. A similar amount of variation i found among haploid *Xenopus* obtained by other means.

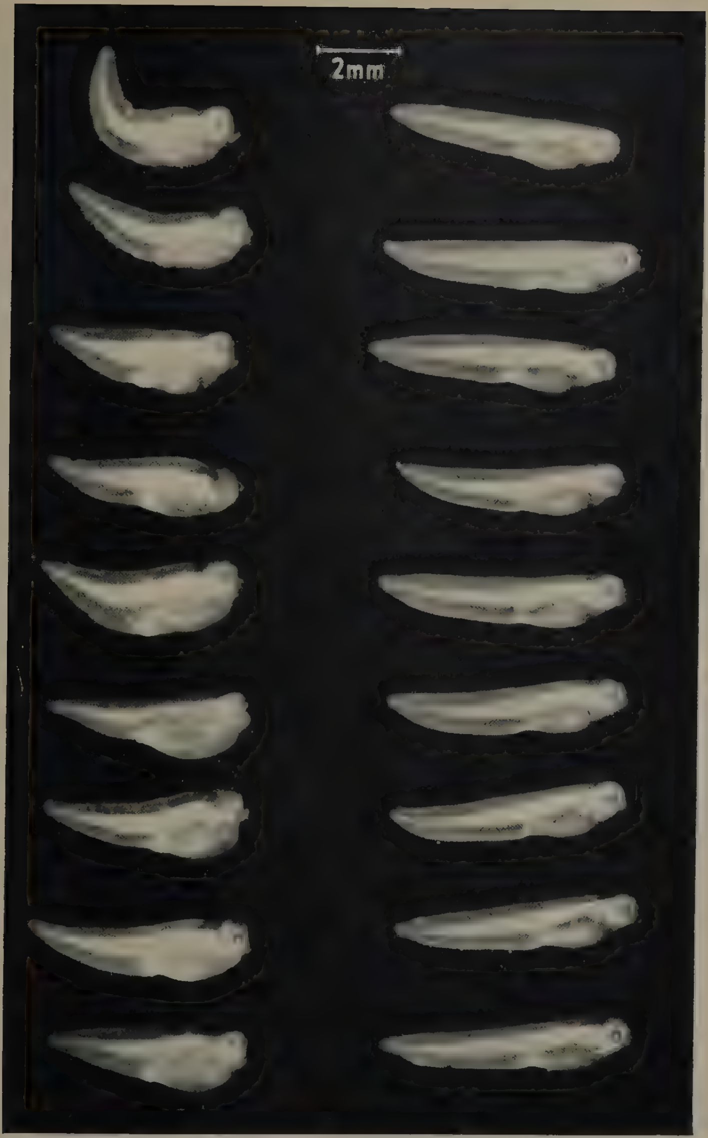

FIG. 4

J. B. GURDON

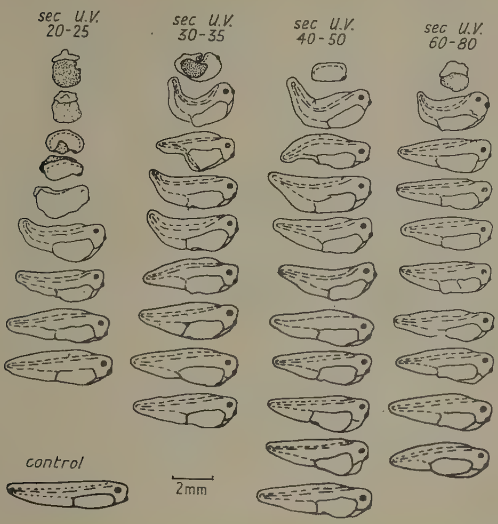

FIG. 5. Camera lucida drawings of haploids resulting from different amounts of u.v. irradiation. Many abnormalities of early development can be seen in the 20 to 25 sec group, while there is a much smaller proportion of early abnormalities in the next three groups.

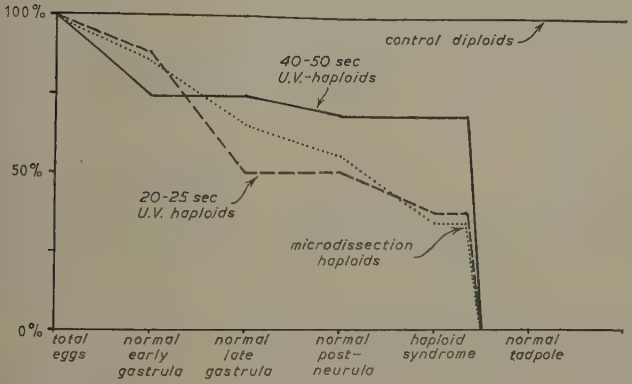

FIG. 6. Survival curves for haploids and controls, based on figures given in table 2. U.v. haploids of the 40 to 50 sec group have a very similar survival curve to those of the 30 to 35 and 60 to 80 sec groups, which are not shown. The method by which haploids are obtained causes different proportions of abnormalities in early development, but the furthest stage of differentiation reached by haploids is always the same.

in the abnormalities shown by u.v. haploids when the u.v. dose is increased from 30 to 80 sec. The egg nucleus appears to have been completely inactivated by 30 sec. The fact that this two to threefold increase in u.v. dose causes no increase in abnormalities is a very strong reason for believing that no cytoplasmic damage was incurred.

The survival of transplant-embryos. The survival of transplant-embryos varies considerably according to the condition of recipient eggs. If an early donor stage with undifferentiated nuclei is used and the recipient eggs are of good quality, it may happen that all transplant-embryos which pass the late blastula stage become normal tadpoles. This shows that at least in these cases the u.v. treatment of the recipient eggs could not have caused any cytoplasmic damage which has any effect after the late blastula stage.

Irradiated fertilized eggs which become diploids. If fertilized eggs are irradiated with u.v. more than 20 min after fertilization, the pronucleus of most eggs has sunk below the surface and is not affected by the u.v. The resulting embryos are therefore diploid and these develop into normal tadpoles. This shows that in these embryos the u.v. causes no cytoplasmic damage.

Eggs which are irradiated for 10 to 20 min after fertilization on the vegetal pole as opposed to animal pole, are diploid and suffer no externally visible abnormalities.

Conclusions of importance for the interpretation of nuclear transplantation experiments

The main purpose of this investigation has been to analyse the effect of u.v. irradiation on unfertilized eggs which were subsequently used for nuclear transplantation. The following comments therefore draw attention to the conclusions from this work which are particularly relevant to the interpretation of nuclear transplantation experiments. An optimum u.v. dose is chosen for the eggs of each female frog, such that a pipette can be pushed into the egg without causing damage or preventing healing. This dose has been found to be nearly double the minimum required to inactivate the egg nucleus completely. This shows that the egg nucleus is very thoroughly inactivated before the egg is provided with a transplanted nucleus. In the case of unfertilized eggs there is no need to irradiate them immediately after laying, since in contrast to fertilized eggs the egg nucleus remains on the surface. When the pronucleus degenerates it appears not to disintegrate into visible fragments, but to disappear gradually as a unit; this makes it unlikely that fragments from the pronucleus become attached to the developing transplanted nucleus and affect the future development of the egg.

Concerning u.v. damage to the egg cytoplasm, it is worth pointing out that in fertilized eggs irradiated for between 40 and 80 sec, only about 8% of eggs showed abnormalities after the late blastula stage (table 2). This means that with the u.v. doses used for nuclear transplantation experiments, most of the damage which might be attributed to u.v., results in arrested late blastulae. For most of our transplantation experiments it is desirable to consider only those transplant-embryos which develop beyond late blastulae. Reasons have already been given (pp. 307–10) for believing that the egg cytoplasm suffers very little u.v. damage with the doses used; but in any case most of the developmental abnormalities which might be attributed to u.v. do not interfere with the interpretation of nuclear transplantation experiments.

I wish to thank Professor Sir A. C. Hardy, F.R.S., for having enabled this work to be carried out in his Department. I am very much indebted to Dr. M. Fischberg for his many helpful suggestions throughout this work and for his attention to the manuscript. I am very pleased to acknowledge the contribution to this work of Dr. T. R. Elsdale, in collaboration with whom the usefulness of u.v. treatment and of other aspects of the technique was first appreciated. Miss A. G. Jewkes has provided most helpful technical assistance during this work.

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The Chorionic Plastron and its Role in the Eggs of the Muscinae (Diptera)

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(*From the Department of Zoology, University of Bristol*)

With one plate (fig. 6)

SUMMARY

In flies of the subfamily Muscinae the egg-shell has both an outer and an inner neshwork layer, each of which holds a continuous film of air. Between these two meshwork layers there is a more or less thick middle layer to which the shell chiefly owes ts mechanical strength. Holes or aeropyles through the middle layer effect the coninuity of the outer and inner films of air. Both meshwork layers consist of struts that rise perpendicularly from the middle layer. In both layers the struts are branched at heir apices in a plane normal to their long axes. These horizontal branches form a fine nd open hydrofuge network that provides a large water–air interface when the egg is mmersed.

When it rains or when the egg is otherwise immersed in water, the film of air held n the outer meshwork layer of the shell functions as a plastron. To be an efficient espiratory structure a plastron must resist wetting by both the hydrostatic pressures nd the surface active materials to which it is normally exposed. The plastrons of all he Muscinae tested resist wetting in clean water by pressures far in excess of any they re likely to encounter in nature. The resistance of a plastron to hydrostatic pressures aries directly as the surface tension of the water, and the surface tension of water in ontact with the decomposing materials in which the Muscinae lay their eggs is much owered by surface active materials. These considerations seem to provide an explanaon for the great resistance of the plastron of the Muscinae to wetting by excess ressures and for the paradox that the plastrons of these terrestrial eggs are more esistant to high pressures than are the plastrons of some aquatic insects that live in ean water.

INTRODUCTION

PREOCCUPATION with the aquatic adaptations of aquatic insects has resulted in an almost total neglect of the aquatic adaptations of terrestrial isects. But whenever it rains heavily a very large number of terrestrial insects e submerged beneath a layer of water. The immobile stages of these insects, ie eggs and pupae, are normally glued or otherwise fastened to the substrate nd necessarily remain submerged until it has stopped raining and the water as evaporated or flowed away. Thus in most climates many of the terrestrial sects are alternately dry and flooded. To be submerged in water for several ours or even days, a period that may, for instance, exceed the duration of the g stage, is no rare and isolated event but is a normal hazard of their environent. It therefore seems likely that many terrestrial insects are adapted for spiration in water in a manner no less complex than are many aquatic sects.

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When the terrestrial environment is seen as one that is alternately dry and flooded, and that when flooded the water is usually well aerated, it is possible to predict the kind of respiratory adaptations that might be expected to be found amongst terrestrial insects. For instance, one of the most characteristic respiratory adaptations of insects that live in aquatic environments that at one moment are flooded by well aerated water and that at another may be dry is the physical gill called a plastron. The great advantage of a plastron in such environments is that when the insect is submerged it provides a relatively enormous water–air interface for the extraction of oxygen from the ambient water, but when the insect is exposed above water the plastron does not involve water-loss over an enormous surface area because the connexion between the plastron and the internal tissues is restricted. The great disadvantage of a plastron is that it becomes an efficient means of extracting oxygen from the tissues should the oxygen pressure of the environment fall below that of the tissues; and it is no accident that the aquatic insects with plastrons are restricted to environments in which the oxygen pressure is maintained at a high level such as streams, the littoral of large lakes, and intertidal areas.

The problems of respiration in aquatic environments liable to sudden drying and in many terrestrial environments are so similar as to suggest that the respiratory adaptation characteristic of the former will be found in the latter. And this is indeed so. A plastron was first reported from terrestrial insect eggs in 1959 (Hinton, 1959), and from more recent work (Hinton, 1960*a*, 1960*b*) it is taking no great risk to predict that examples of plastron respiration amongst terrestrial insects will be found to be much more numerous than amongst aquatic insects.

The eggs of all oviparous species of Muscinae examined have a plastron. The principles of plastron respiration have been summarized by Thorpe (1950). The hydrostatic pressures that have to be applied in order to wet the plastron of the Muscinae are considerably greater than any to which they are at all likely to be exposed in nature. In this respect they resemble the plastrons of other dipterous eggs that are also laid in decaying organic matter. Not only do the plastrons of such eggs resist wetting by hydrostatic pressures far in excess of any they are likely to encounter, but those of some species are even more resistant to excess pressures than those of many aquatic insects. For instance, the plastron of the terrestrial egg of *Drosophila funebris* F. resists wetting by an excess pressure of 1·3 atm (Hinton, 1960*a*), whereas that of the aquatic pupa of *Taphrophila vitripennis* Meig. only resists about 0·3 atm (Hinton, 1957).

Eggs laid in decaying organic matter are probably often exposed to concentrations of surface active substances that rarely if ever occur in streams. For instance, the surface tension of the temporary pools of rain water on cow pats is reduced to about 50 dyn/cm, and under comparable conditions the surface tension of water on the surface of decomposing flesh is reduced to about 40 dyn cm (Hinton, 1960*a*). To be an efficient respiratory structure a plastron must resist wetting by surface active substances. Any change in the geometry or the nature of the surface of the plastron meshwork that increases its resistance to wetting by surface active substances also increases its resistance to wetting in clean water by excess pressures, since wetting by excess pressures always occurs before there is a mechanical breakdown of the plastron meshwork. Therefore selection for greater resistance to wetting by surface active substances inevitably results in greater resistance to hydrostatic pressures; and this fact provides an explanation of the paradox that the plastrons of some terrestrial insects resist higher hydrostatic pressures than those of some aquatic insects.

A comparative account of the respiratory structures of the egg-shell of the 10 genera of the subfamily Muscinae that occur in Britain is given here. Details of the respiratory system of the shell have previously been given for only one species in the subfamily, *Musca* (*Eumusca*) *autumnalis* Deg. (Hinton, 1960*a*). To reduce description of morphological detail, the structure of the shell and the respiratory system of what is conceived to be the primitive egg of the Muscinae is given; and under the headings of the different species only the way in which these structures are modified is noted. The oviposition habits of each species are briefly summarized; they assist in understanding the adaptive significance of differences between the species in the structure of the shell and respiratory system.

MATERIALS AND METHODS

Most of the eggs used were collected in the field or bred in the laboratory. The identity of eggs collected in the field was established by comparison with eggs laid by isolated females in the laboratory. The eggs of *Pyrellia*, *Stomoxys*, and *Lyperosia* were dissected from dried museum specimens, and those of *M. domestica* were obtained from cultures kept in the Pest Infestation Laboratory at Slough.

The structure of the respiratory system was examined by means of whole mounts and serial sections. In most species the branches of the network of both the outer and inner surfaces of the shell are only resolved with the light microscope by the most careful adjustment and the use of a blue filter: most of the branches that are normal to the vertical struts exceed the wavelength of visible light in only one dimension. Electron micrographs of the respiratory system of the egg of the fly *Dryomyza flaveola* F. (Hinton, 1960*a*) revealed the fact that all essential features could be resolved with the light microscope.

A column of water was used to study the resistance of the plastron to a hydrostatic pressure of 7 cm Hg. Its resistance to higher pressures was tested in a chamber connected to a mercury reservoir by a long piece of rubber tubing. The pressure required was obtained by raising the mercury reservoir the appropriate distance above the chamber. In all experiments live eggs were used. The figures for the resistance of the plastron to excess pressures are therefore minimum figures. Any oxygen uptake by the egg must produce a pressure gradient in the plastron and therefore a fall in the back pressure of the system. The maximum possible fall in the back pressure of the system due to the respiration of the egg would be equivalent to a rise in the hydrostatic pressure of about 16 cm Hg. However, as in all experiments the total volume of the eggs used was small in comparison to that of the well-aerated tap-water in the chamber, and most experiments lasted only ½ h, it is probable that the actual hydrostatic pressures were at most only a few centimetres greater than those recorded.

BASIC STRUCTURE OF RESPIRATORY SYSTEMS OF EGG-SHELL

Ten genera of the subfamily Muscinae occur in Britain: *Musca*, *Orthellia*, *Dasyphora*, *Pyrellia*, *Mesembrina*, *Morellia*, *Polietes*, *Stomoxys*, *Haematobia*,

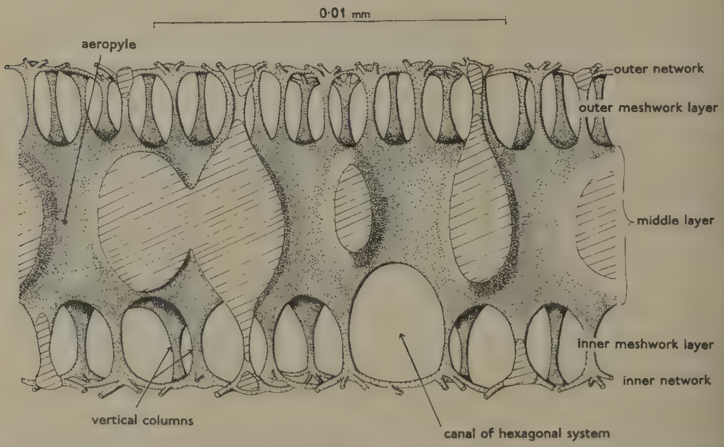


FIG. 1. Diagrammatic view of a section through the egg-shell of a 'typical' member of the Muscinae, to show the relations of parts. In the illustrations that follow, the cut edges of both outer and inner networks are drawn as more or less solid lines: with the light microscope it is only possible to resolve the branches of these networks when they are seen from a view more or less normal to the plane of the surface.

and *Lyperosia*. The respiratory system of each of these genera conforms to an easily recognizable pattern that differs greatly from that found in other subfamilies of the Muscidae. The chorion (fig. 1), including the area between the hatching lines, always has both an outer and an inner meshwork layer, each of which holds a continuous layer of air. Between these two layers there is a more or less thick middle layer to which the shell chiefly owes its mechanical strength. Holes or aeropyles through the middle layer effect the continuity of the outer and inner layers of air. In some genera these aeropyles may be as much as 3 μ wide, whereas in others they are never wider than about 0·2 μ.

The outer meshwork layer consists of struts which arise perpendicularly from the outer surface of the middle layer. In any one species these struts are all of about the same length. The struts are branched at their apices in a plane normal to their long axes. These horizontal branches connect the apices of adjacent struts and also sometimes anastomose with one another. They form a fine and open hydrofuge network at the surface of the shell. When the egg is immersed in water, the air–water interface of the plastron is confined to the interstices of the surface network of the shell.

The inner meshwork layer consists of struts that extend vertically inwards from the inner surface of the middle layer. They correspond to the structures that in other eggs are known as the vertical columns. In any one species the vertical columns are usually of about the same length, but they may be longer or shorter or equal in length to the struts of the outer meshwork layer. They are arranged in somewhat irregular hexagons, the boundaries of which correspond to the boundaries of the follicular cells. At the boundaries of the hexagons the vertical columns are absent, so that there is a continuous hexagonal network of air canals. The middle layer of the chorion is here also more deeply excavated than elsewhere with the result that in sections the canals of the hexagonal system are easily recognizable as distinctly higher and wider spaces in the inner meshwork layer (fig. 1). When the outer surface of the middle ayer is ridged, the ridges are developed along the boundaries between follicular cells. Thus the shell may have a superficial hexagonal pattern of ridges hat is widely separated from but directly above the air canals of the inner meshwork layer.

The presence of an outer meshwork layer in the shell of all 10 genera of Muscinae examined supports the view that this layer is a primitive feature of he subfamily. In other subfamilies of the Muscidae (Hinton, 1960*a*) and in families related to the Muscidae, such as the Calliphoridae (Hinton, 1960*b*) and Corduluridae (Hinton, 1960*a*), as well as in the Schizophora generally, he chorion has only an inner layer of meshwork. In such eggs there is only one instead of two continuous layers of air in the shell outside the hatching ines, and there would seem to be little doubt that this is the primitive condiion of the terrestrial eggs of the higher Diptera. Thus, although the possession of two meshwork layers is a primitive feature of the subfamily Muscinae, it is nevertheless a specialized, secondary feature within the family Muscidae.

A further characteristic feature of the eggs of the Muscinae, previously noted by Collin (1948), is that the chorion on each side of the hatching lines s apparently never elevated to form a high ridge or flange. The genera *Graphomyia* and *Muscina* were transferred by Collin from the Muscinae to he Phaoniinae because of the presence of flanges, and the genus *Polietes* was ransferred from the Phaoniinae to the Muscinae because the egg lacked langes. The structure of the respiratory system of the chorion of these three genera fully supports the view of Collin.

On the basis of adult structures, some writers erect a separate tribe or subfamily to contain *Stomoxys*, *Haematobia*, and *Lyperosia*. The respiratory ystems of these three genera are chiefly remarkable for their very long and narrow aeropyles (fig. 9, A, C, p. 330), which are in sharp contrast to the very hort and broad aeropyles of *Musca* and related genera. The structure of the respiratory system of *Polietes* (fig. 8, B, p. 328), however, is intermediate between these two groups of genera, although the adults of *Polietes* lack many of the features that characterize *Stomoxys* and its allies.

In the primitive Muscid egg the outer part of the shell consists of a thick and solid sheet of chorion. Direct communication between the ambient air and the inner layer of air in the shell is restricted to the area of the hatching lines. The relative importance of the shell and sub-choral membranes in restricting water loss is not known. However, in the Muscinae the structure of the shell is such that it cannot restrict water-loss except in the sense that it holds a stationary layer of air around the embryo and so establishes a thick diffusion boundary layer. Since the shell of the Muscinae consists in effect of a thick but open meshwork (fig. 1), it may be supposed either that it evolved in an environment in which the egg was not likely to be dried out during its incubation period, or that it evolved simultaneously with an increase in waterproofing of the sub-choral membranes. Of these two hypotheses, the former appears much more likely because the waterproofing properties of the subchoral membranes are slight, as is evident from the fact that the eggs become too dry to develop or hatch even in moist air. For instance, Larsen (1943, fig. 6) has shown that at 25° C the eggs of *Haematobia* and *Lyperosia* fail to hatch when the relative humidity is 83%, and less than 15% of the eggs of the house fly and *Stomoxys* hatched at an r.h. of 80%. Even at an r.h. of 93% to 94% only 5% of the eggs of *Lyperosia* hatched. The incubation period of the eggs of these flies is well below a day at optimum temperatures. For instance, at an r.h. of about 100%, the incubation period of the house-fly egg is about 7 h at 35° C and about 14 h at 25° C.

Except for *Musca* (*Eumusca*) *autumnalis*, all the oviparous species in the 10 genera here considered lay in or on very moist dung or other moist organic matter that is very unlikely to dry out before the eggs hatch. The eggs of *M. autumnalis* are laid singly, each in a narrow hole made by the female in the upper surface of the dung. The egg stands vertically in the hole. The respiratory horn often projects slightly above the surface, and generally neither it nor the apex of the egg is in contact with the dung. This egg is thus more exposed to dehydration than that of any of the other Muscinae known to me. It is therefore of considerable interest to note that the outer surface of the shell, apart from the area between the hatching lines, is not an open network but is a continuous sheet of chorionin. Nevertheless, the shell of this egg still retains the outer meshwork layer of the Muscinae, which, as in other Muscinae, holds a continuous layer of air. Aeropyles through the middle layer of the shell effect the continuity of the two layers of air in the shell of *M. autumnalis* just as they do in other Muscinae. Amongst the Muscinae, the egg of *M. autumnalis* and those of other species of the subgenus *Eumusca* are highly specialized not only in having respiratory horns but also in having a solid outer layer of chorionin. In other Muscidae, e.g. Phaoniinae, the solid outer layer of chorionin is a primitive feature, whereas in the species of *Eumusca* it is a specialized, secondary one.

Orthellia caesarion Meig.

The egg (fig. 3, B) is 2·1 to 2·2 mm long and 0·5 mm wide. There is a con-picuous cap of cement covering the posterior tenth or so and another very nuch smaller cap over the micropylar area. The hatching lines extend nearly o the posterior pole and are everywhere parallel except near the apex, where hey diverge slightly as shown in fig. 3, B. The outer meshwork layer is slightly hicker than the inner in most parts of the shell (fig. 2, B), but usually near the atching lines it is very much thicker (fig. 2, A). The aeropyles (fig. 2, C) are sually 1·0 to 1·6 μ broad. The chorion above the canals of the hexagonal stem is not raised, and its outer surface therefore appears smooth at magnifi-tions that do not resolve the outer network. The structure of the chorion etween the hatching lines (fig. 2, A) is very similar to that close to but outside e hatching lines. A small area at the posterior pole has wide canals in the ter meshwork layer. These canals are arranged in an hexagonal pattern that es directly above the canals of the inner meshwork layer, and throughout eir length the outer canals are connected to the inner ones as in *Morellia* g. 4, C). The posterior cap of cement completely covers the area in which nals are present in the outer meshwork layer.

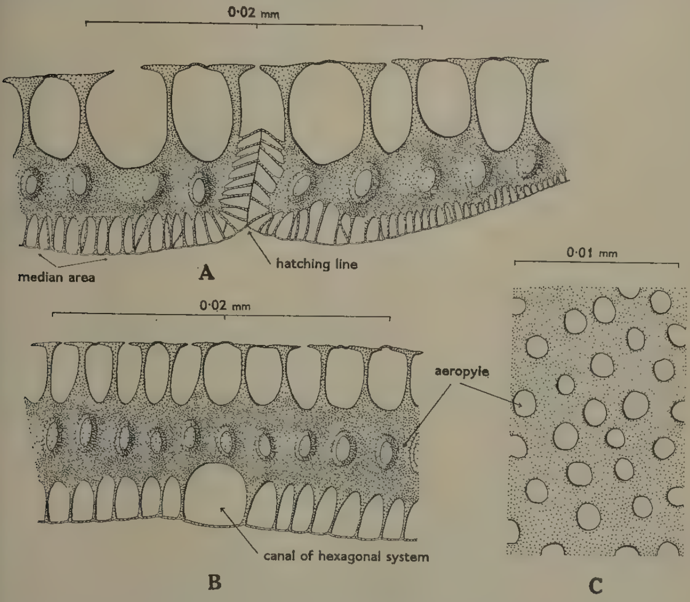


FIG. 2. *Orthellia caesarion.* A, transverse section of the chorion on each side of the hatching line. B, transverse section of the dorso-lateral chorion of the middle of the shell. C, optical section through middle layer of aeropyles of dorso-lateral area of middle of shell.

Paraffin oil applied to any part of the inner surface network immediately enters the inner meshwork layer and runs through the aeropyles into the outer meshwork layer, displacing the air in both layers. When the eggs had been kept in water for a short time, oil immediately penetrated through the outer surface network and displaced the air in both layers. When oil was applied to the outer surface of fresh eggs that had not been in contact with water, it nearly always penetrated through the network of the median area, but in about 50% of the eggs tested did not penetrate or spread on many parts of the surface of the remainder of the shell. The cause of this irregularity was not determined, but it may have been due to the presence sometimes of a very thin film, perhaps of cement, on the surface. The surface of dry unwashed eggs sometimes has a slight metallic sheen not present in dry washed eggs.

O. caesarion begins to arrive on cow-pats about a minute or so after they are deposited, and, as pointed out by Hammer (1941), the greatest number are found on cow-pats 30 to 60 min old. A few have been seen to lay in cow-pats 1 to 2 days old. The eggs are laid in cavities that are almost always in the upper surface of the dung. The cavities are usually 1 to 3 mm below the surface. When the dung is very fresh, the fly usually lays in the crests of ridges, perhaps because these areas are the first to dry and form a slight crust. As the dung becomes older and drier, the eggs are more frequently laid in depressions on the surface. The cavity is constructed with the aid of the ovipositor alone. Each egg is attached vertically to the floor of the cavity by a cement that forms a cap over the posterior tenth of the egg (fig. 3, B). The first egg of each batch is laid directly below the hole into the cavity when the latter is only a little wider than this hole. As further eggs are laid around the first, the cavity is gradually enlarged, its walls being pressed away from the centre by the ovipositor. The eggs are laid in close circles around the first egg so that each is in contact with its neighbours (fig. 3, A). When the dung is semi-liquid and its consistency more or less even, the floor of the cavity is more or less circular. However, especially when the dung is drier, groups of fibres may resist pressure from the ovipositor so that the shape of the cavity may be irregular. After the eggs have been laid and the ovipositor has been withdrawn, the entrance to the cavity remains open. The entrance holes are 1 to 3 mm long. Their diameter varies from 0·6 to 1·1 mm, and the average of 11 was 0·89 mm.

The number of eggs in a batch was usually 25 to 35, but as many as 41 have been found, and a few cavities contained only 1 to 5 eggs. The great variation in the size of the batches is perhaps chiefly due to the fact that when the female is disturbed sufficiently to abandon the site she does not return again to lay in it but constructs a cavity elsewhere for the remainder of her eggs. According to Hammer (1941), several females will sometimes lay in the same cavity, so that batches of 80 to 150 or more eggs are common, but I have not seen such batches. The eggs hatch in 1 to 3 days according to the temperature. Upon hatching either the right or the left hatching line splits completely, the other hatching line remaining intact or splitting only along its anterior tenth or so.

When it rains or when water is poured over the cow-pat, water is prevented om entering the cavity or the entrance hole by the trapped air. The bubble f air so trapped not only functions as an air store but must also function as temporary physical gill. Oxygen withdrawn from the bubble lowers the artial pressure of the oxygen and raises the partial pressure of the nitrogen the bubble. Equilibrium tends to be restored by oxygen diffusing into the ubble rather than by nitrogen diffusing out of it because of the much greater lubility of oxygen. Nevertheless, nitrogen is continuously diffusing out, and time the size of the bubble will be sufficiently reduced to permit water to ter the cavity. If this should occur, a smaller bubble of air trapped in the aces between the hydrofuge surfaces of the eggs will function as a temporary ysical gill. That a smaller bubble of air is so trapped has been demonstrated enlarging the entrance hole sufficiently to permit the cavity to fill with ater.

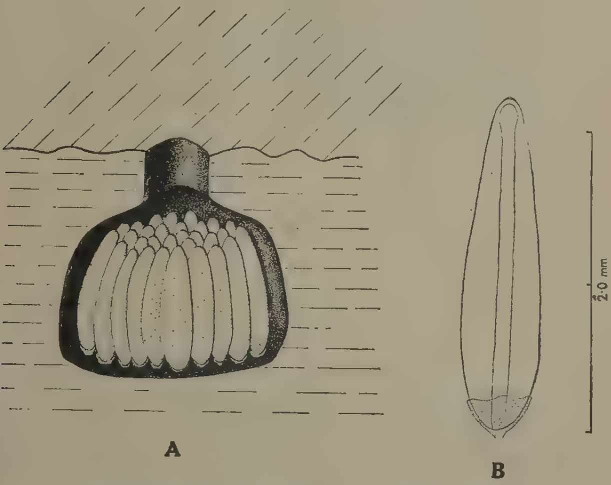


FIG. 3. *Orthellia caesarion.* A, egg cavity in cow-dung cut open to expose the batch of eggs; semi-diagrammatic. B, dorsal view of egg.

When a bubble of air is trapped in the cavity, only a small part of its surface ea is in contact with the well-aerated water at the surface, and therefore the erall role of the bubble will depend upon gas exchanges at the air–dung terface. Thus, if the oxygen pressure in the dung should fall below that in e tissues of the eggs, oxygen will be removed from the eggs. However, it is obable that the latter could only happen for a short time after the cow-pat deposited, since it quickly dries and becomes well aerated by the tunnelling tivities of beetles and other insects.

The resistance of the plastron between the hatching lines to excess pressures is shown in table 1. The resistance of the plastron outside the hatching lines to excess pressures is somewhat greater.

Owing to the manner in which the eggs are placed in a cavity in the dung, they are enveloped by a bubble of air when it rains. At first sight, therefore, the plastron of the shell would appear to have no selective value. However, observation in the field shows that the cavities are often partly destroyed before the eggs hatch by the tunnelling of beetles and other insects. This occurs more frequently when the cavities are formed in older cow-pats. On several occasions cavities have been seen broken open and the batch of eggs exposed as the result of cattle stepping on the cow-pat. Under these conditions the eggs may be in direct contact with water when it rains.

TABLE 1

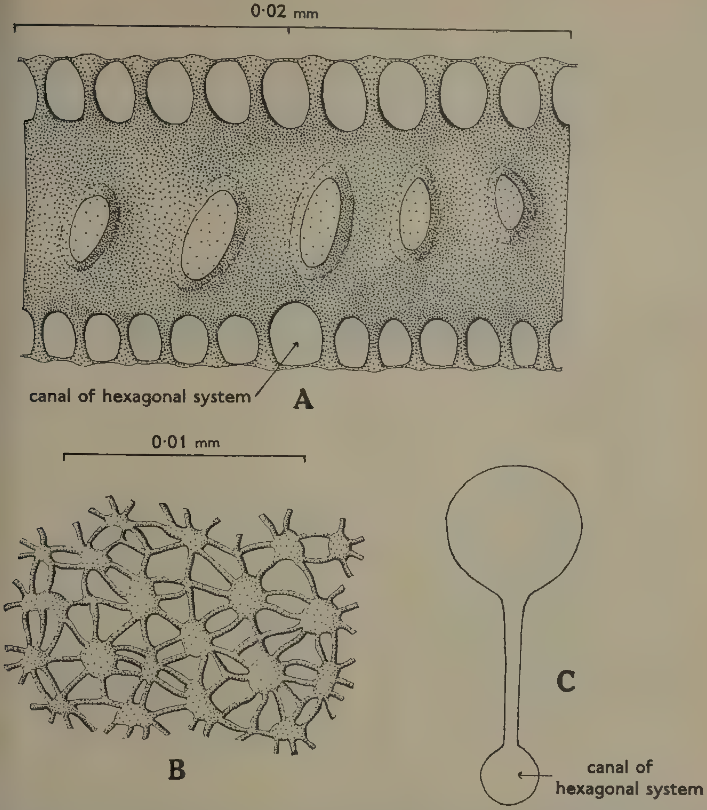
Effects of excess pressures upon the retention of the plastron gas of Orthellia caesarion *(18° to 20° C)*

No. of eggs	*Pressure*	*Time*	*% retaining over 90% of plastron gas*
	(cm Hg)	(h)	
10	7	24	100
15	14	24	80
10	30	½	100
10	30	½	90
12	40	½	75
10	40	½	70
17	60	½	53
10	60	½	40
10	76	½	20
18	76	½	0

The respiratory system of the egg of *O. cornicina* F. appears to be indistinguishable from that of *O. caesarion*, and no other structures have been found that enable me to distinguish the eggs of the two species. The oviposition habits of the two species are similar.

Morellia hortorum Fall.

The egg is 2·1 to 2·2 mm long and 0·5 mm broad. The structures of the shell and respiratory system appear to be identical with those of *Orthellia*, and, as in the latter genus, a small area at the posterior pole has wide canals in the outer meshwork layer (fig. 4, C). The relative thicknesses of the middle and meshwork layers vary slightly in different parts of the shell as they do in *Orthellia*. Hammer (1941) claims that the egg of *M. hortorum* is only 1·5 to 1·6 mm long, but the eggs examined by me were obtained from isolated females and their identity therefore seems certain. The oviposition habits are not known to me, but, according to Hammer, the eggs of the species of *Morellia* are laid in cavities in the dung as are those of *Orthellia*. *Morellia* generally arrives on cow-pats long after *Orthellia*.



G. 4. *Morellia hortorum.* A, longitudinal section of dorso-lateral chorion of middle of shell. surface view of outer network. C, connexion between one of the large canals that form the xagonal pattern in the outer meshwork layer of the posterior pole and one of the smaller canals of the hexagonal system of the inner meshwork layer.

usca domestica L.

The egg is 1·1 to 1·2 mm long and 0·25 to 0·30 mm wide. The hatching es extend from near the anterior to near the posterior pole and except at ch end are parallel. The outer meshwork layer is slightly thicker than the ner in most parts of the shell (fig. 5, A). The aeropyles (fig. 5, C) vary from out 0·6 to 1·0 μ in breadth. The chorion above the canals of the hexagonal stem is not raised, and its outer surface therefore appears to be evenly convex magnifications that do not resolve the outer surface network. The structure the chorion of the median area between the hatching lines is similar to that the chorion outside the hatching lines. There are no wide canals in the outer meshwork layer of the posterior pole as there are in *Orthellia* and *Morellia*.

Paraffin oil applied to any part of the inner or outer surface networks immediately enters the nearest meshwork layer and flows through the aeropyles into the other meshwork layer, displacing the air in both layers.

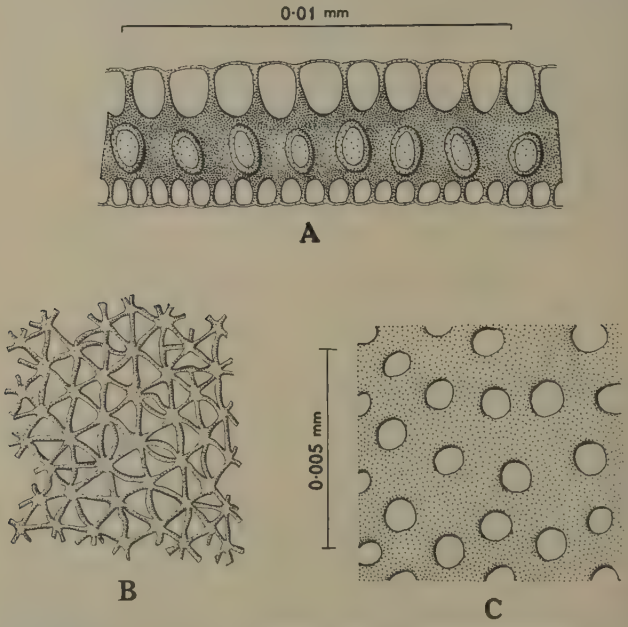


FIG. 5. *Musca domestica*. A, longitudinal section of dorso-lateral chorion of middle of shell. B, surface view of outer network. C, optical section through middle of aeropyles of dorso-lateral area of middle of shell.

The common house-fly has been recorded as breeding in a very wide variety of decomposing animal and vegetable substances, but nearly all writers are agreed that it prefers to lay in pig or horse manure (Patton, 1931; West, 1951). The eggs are usually laid in closely packed batches, each egg being placed vertically upon its posterior end. When the larva hatches, the hatching line of either the right or left side is entirely or nearly entirely split, whereas the split on the hatching line of the opposite side is usually confined to about the anterior eighth.

The resistance of the plastron of the median area between the hatching lines to wetting by excess pressures is shown in table 2. The resistance of the plastron outside the hatching lines to excess pressures is of the same order. The resistance of the plastron to hydrostatic pressures and, since one is a ieasure of the other, to wetting by surface-active substances is unexpectedly ›w in view of the fact that oviposition is recorded in such a wide variety of ecomposing animal and vegetable substances. In the cow dung species, *Iusca* (*Eumusca*) *autumnalis* Deg. (Hinton, 1960*a*), the resistance of the lastron at 30 cm Hg is similar to that of the house-fly at 14 cm Hg. Of all the Iuscids tested, only the plastron of *Myospila meditabunda* F. (subfamily haoniinae), a cow-dung species with respiratory horns, has proved to be less esistant than that of the house-fly.

TABLE 2

Effects of excess pressure upon the retention of the plastron gas of Musca domestica (16° *to* 17° *C*)

No. of eggs	*Pressure*	*Time*	*% retaining over 90% of plastron gas*
	(cm Hg)	(h)	
12	7	24	92
10	14	½	100
11	14	½	91
32	14	½	84
10	14	½	80
12	20	½	66
10	20	½	50
10	30	½	30
10	30	½	20
10	30	½	20
10	40	½	20
10	60	½	0

asyphora cyanella Meig.

The egg is 2·3 to 2·5 mm long and 0·5 wide. The hatching lines extend to ›out the posterior tenth and are close together and parallel throughout except ear the posterior end, where they converge slightly, and near the anterior d, where they diverge. The outer and inner meshwork layers are approxiately equal in most parts of the shell; the outer meshwork layer on each side the hatching lines (fig. 6, D) has the struts that are normal to the plane of the rface about 3 times as long as the vertical struts of the inner meshwork layer; ost of these long struts branch repeatedly before their apices. The aeropyles e usually about 1 μ broad, but, especially in the anterior half of the shell, ere are aeropyles 1·5 to 1·8 μ broad above the corners of the hexagons. The orion above the canals of the hexagonal system is slightly convex.

The eggs are laid in large batches just under the upper surface of cow-pats. nfortunately, the precise nature of the cavity in which they are laid was not ted on the only occasion that eggs were found. According to Thomson 937), 'The eggs are laid in a manner very similar to that of *Cryptolucilia* *rthellia*] in batches of 25–30 just under the surface of the dung'.

Pyrellia cyanicolor Zett.

The egg is known to me only from specimens dissected out of dried females. The structure of the chorion and respiratory system appears to be identical to that of *Dasyphora cyanella*. I do not know where the egg is laid or on what the larva feeds.

Mesembrina meridiana L.

The egg is 4·0 to 4·3 mm long and 1·1 to 1·3 mm wide. The hatching lines are close together and more or less parallel, diverging slightly at the anterior end and converging at the posterior end. Outside the hatching lines the inner meshwork layer is usually 7 to 9 times as thick as the outer meshwork layer (fig. 7, B). In the median area between the hatching lines the inner meshwork layer is often only 2 to 3 times as thick as the outer. The aeropyles are about as long as wide and are often $2\,\mu$ or more wide. The canals that form the hexagonal pattern are very wide in both the median area (fig. 7, A) and the area outside the hatching lines (fig. 7, B). The chorion of the middle layer is not, or not regularly, raised above the canals of the hexagonal system.

Paraffin oil applied to any part of the outer or inner surface of the shell, except in the area of the micropyle, immediately enters the nearest meshwork layer and flows through the aeropyles into the other meshwork layer, displacing the air in both layers. As the oil is withdrawn, air first enters into the canals of the hexagonal system and only much later into the meshwork between the canals.

Mesembrina arrives on the cow-pat within a few minutes of its deposition, and the greatest number are found on cow-pats about 15 min old, as noted by Hammer (1941). A single egg is laid at a time. It is pushed more or less vertically into the dung, but its anterior fifth or so is left projecting above the surface of the dung. The larva hatches almost immediately, sometimes as soon as the female has withdrawn its ovipositor. When the larva hatches, the hatching line of either the right or the left side is entirely or nearly entirely split open, whereas the split on the hatching line of the opposite side is usually confined to about the anterior third. The incubation period is passed in the common oviduct. Sometimes the first larval moult occurs while the egg is in the common oviduct, and it is then the second larval instar that hatches from the egg (Thomson, 1937). Although several eggs may be found in a single cow-pat, no female lays more than one in any particular pat, because by the time another egg is mature the cow-pat is too old.

Not only is the egg of *Mesembrina* laid in a position more exposed to desiccation than that of any other Muscinae, but the structure of the shell is

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FIG. 6 (plate). A, transverse section of dorso-lateral chorion of *Mesembrina meridiana*.

B, transverse section of median area between hatching lines of the same species.

C, aeropyles of *Orthellia caesarion*. The aeropyles appear more unequal in width than they in fact are (see fig. 2, C) because some are partly out of focus.

D, median area beside the hatching line of *Dasyphora cyanella*, showing struts of the upper meshwork layer that are branched before their apices.

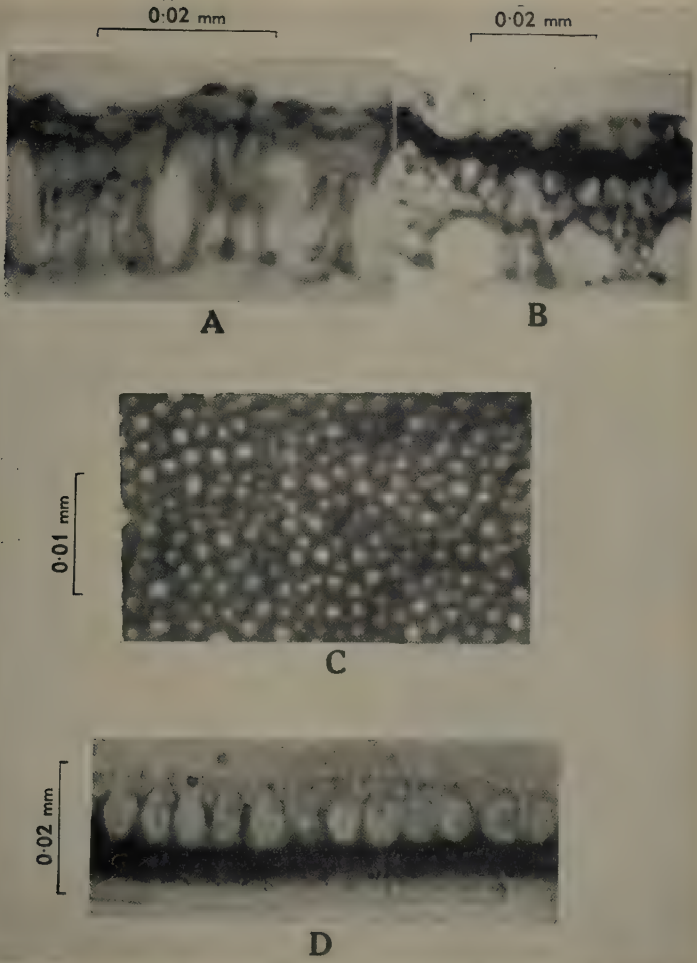

FIG. 6
H. E. HINTON

ess adapted than any to resist water loss. However, the egg-shell functions utside the body of the female for only a few seconds or minutes, as the species s very nearly ovo-viviparous.

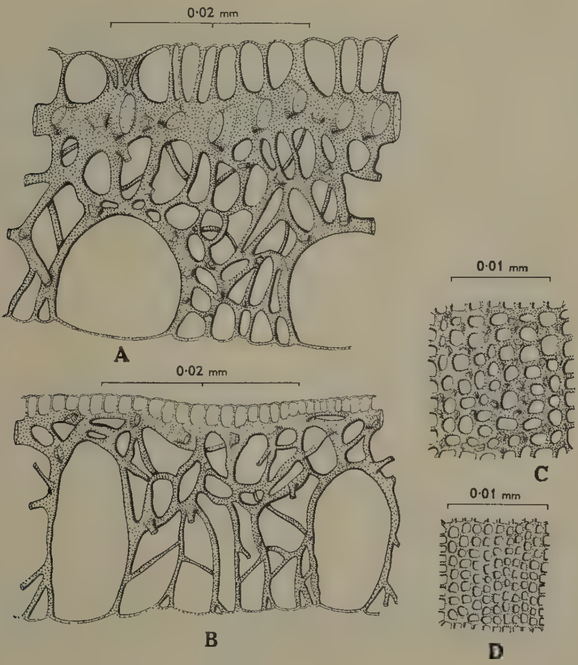

FIG. 7. *Mesembrina meridiana.* A, transverse section of a part of the median area between the hatching lines. B, transverse section of the dorso-lateral chorion of the middle of the egg. C, optical section of upper surface of aeropyles of middle dorso-lateral area of shell. D, surface view of inner network of shell.

olietes lardaria F.

The egg is 2·2 to 2·3 mm long and 0·67 to 0·75 mm wide. The form of the atching lines is shown in fig. 8, A. The outer meshwork layer is usually icker than the inner (fig. 8, B). The aeropyles (fig. 8, B, C) are usually 0·3 to ·6 μ wide and are usually about 30 times as long as wide. The middle layer f the shell is distinctly convex above the canals of the hexagonal system, so at on the outer surface of the egg there is an hexagonal pattern of low but istinct ridges that are conspicuous at a magnification of ×75. The structure f the median area between the hatching lines is similar to that of the chorion sewhere, but the pattern of hexagonal ridges is finer and less distinct.

Paraffin oil (sp. gr. 0·88–0·89) applied to the inner surface of the shell

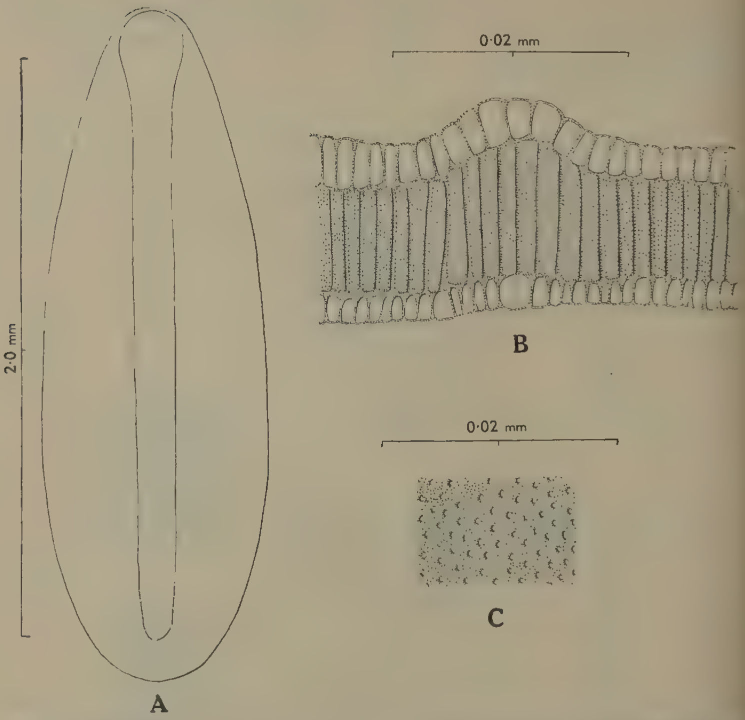

FIG. 8. *Polietes lardaria*. A, dorsal view of egg. B, longitudinal section of dorso-lateral chorion of middle of shell. C, optical section through middle of aeropyles of dorso-lateral area of middle of shell.

immediately enters and displaces the air in both meshwork layers. Oil applied to the outer surface of the shell of dry eggs that had previously been lightly washed in water behaves in the same way. However, paraffin oil does not penetrate nor spread upon portions of the outer surface of shells that have neither been rubbed nor wetted with water. Rubbing the outer surface very gently with a delicate wisp of paper is sufficient to cause oil to penetrate, as is washing it lightly in tap-water. It thus appears that, as in *Orthellia*, the surface of fresh eggs is coated with a very thin lipophobe, water-soluble substance. It is hoped to investigate this film in more detail when sufficient eggs are available.

Polietes sometimes arrives within a few minutes of the cow-pat being deposited, but most arrive when the cow-pat is older than 15 min. The eggs are usually laid in small batches on the surface of the lower sides of the pat or even on the ground or near the ground on grass or grass roots near the pat. The oviposition habits of *Polietes hirticrura* Meade are similar (Thomson, 937). The humidity of the air at the bottom or in the lower part of the layer f grass near the cow-pat is probably normally close to saturation. Eggs which ad begun to shrivel, as they do when exposed to relative humidities below o%, were never found in the field. The resistance of the plastron of the edian area to wetting in tap-water by hydrostatic pressures is shown in ble 3. The resistance of the plastron outside the hatching lines is similar.

TABLE 3

Effects of excess pressures upon the retention of the plastron gas of Polietes lardaria (18° *to* 20° *C*)

No. of eggs	*Pressure*	*Time*	% *retaining over* 90% *of plastron gas*
	(cm Hg)	(h)	
10	7	24	100
10	30	½	100
8	40	½	100
10	40	½	40
6	60	½	40
10	60	½	20
10	60	½	10

aematobia stimulans Meig.

The egg is 1·5 to 1·6 mm long and 0·44 mm wide. The form of the hatching es is as in *Polietes* (fig, 8, A) but the lines are wider apart in relation to the ze of the egg, being separated by 0·15 mm in the middle of the egg. Most the aeropyles are less than 0·2 μ wide, but at positions corresponding to the rners of the hexagons made by the wide canals in the inner meshwork layer ropyles about 1 μ wide are present (fig. 9, A, B). The inner meshwork layer distinctly thicker than the outer (fig. 9, A). The middle layer of the shell is stinctly convex above the canals of the hexagonal system, so that on the ter surface of the egg there is an hexagonal pattern of low but distinct dges that are conspicuous at a magnification of ×75. The structure of the edian area between the hatching lines is similar to that elsewhere. That the ner and outer surfaces of the chorion generally, as well as the surfaces of e area between the hatching lines, consist of an open network has been nfirmed by direct observation and also by the fact that oil applied anywhere the inner or outer surfaces, except in the region immediately around the icropyle, displaces the air in the meshwork layers.

Haematobia usually arrives on the cow-pat within the first minute of its position, and it is rarely found on cow-pats as much as 20 min old (Hammer, 41). The eggs are laid singly or in small groups. They are sometimes laid crevices on the upper surface, but most are laid on the lower sides of the t or on the ground near the pat. The oviposition habits of this species thus semble those of *Polietes*. When the larva hatches, it splits the entire hatching e of either the right or left side, the hatching line of the opposite side being

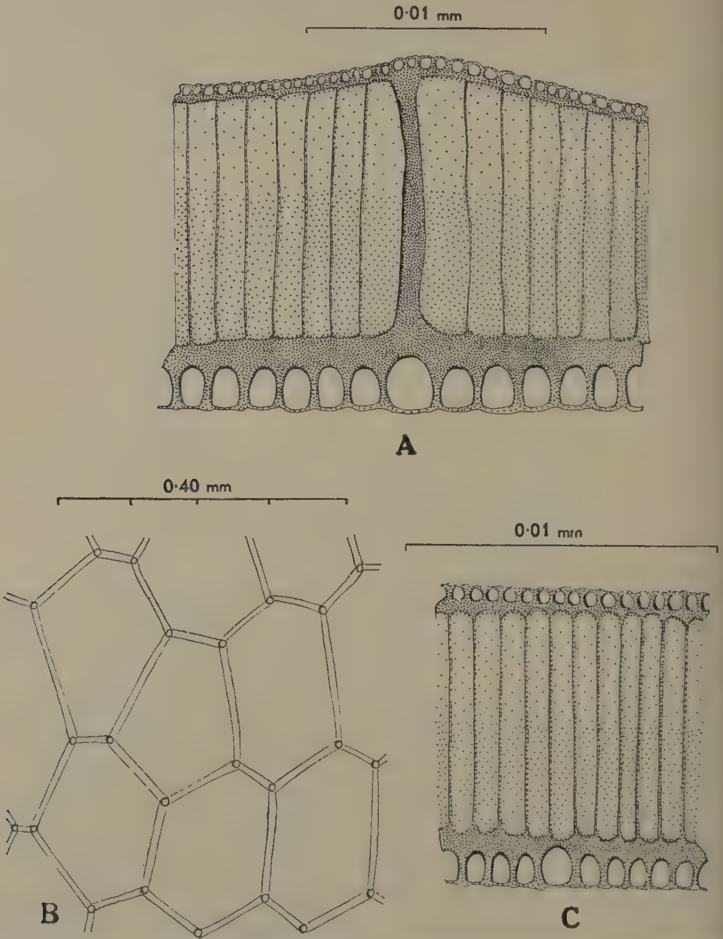

Fig. 9. A, longitudinal section of dorso-lateral chorion of middle of shell of *Haematobia stimulans*. B, relation of coarse aeropyles to canals of the hexagonal system of the same species. C, longitudinal section of dorso-lateral chorion of middle of shell of *Stomoxys calcitrans*.

split only anteriorly. Only a few eggs were available for experiments. Over 90% of the plastron of the median area between the hatching lines remained intact in 4 eggs subjected to a hydrostatic pressure of 30 cm Hg for 30 min and in 3 eggs subjected to 40 cm for the same period. Of 5 eggs subjected to 60 cm for 30 min over 90% of the plastron was intact in 4. The resistance of the plastron outside the hatching lines to wetting by hydrostatic pressure was found to be similar.

Stomoxys calcitrans L.

The egg is known to me only from specimens dissected out of dried females. t is about 1 mm long and 0·3 mm wide. The structure of the respiratory ystem is very similar to that of *Haematobia*, but the aeropyles over the canals f the hexagonal system of the inner meshwork layer are no wider than the eropyles elsewhere (fig. 9, C). The outer surface of the middle layer of the horion is not distinctly raised above the canals of the hexagonal system.

According to Thomson (1937), the eggs are laid in batches of 5 to 20 in racks in the crust of middens containing cow-, horse-, or pig-dung. Fresh ung is generally avoided. In cattle stalls the eggs are laid in litter mixed with ung and urine.

yperosia irritans L.

The egg is known to me only from specimens dissected out of dried females. t is 1·2 mm long and about 0·3 mm wide. The structure of the respiratory ystem appears to be identical to that of *Stomoxys*. The egg may be readily istinguished from that of the latter species because the outer surface of the iddle layer of the chorion between the hatching lines is raised in the form of istinct ridges above the canals of the hexagonal system of the inner meshwork ayer.

According to Hammer (1941), the females arrive immediately the cow-pat s deposited and often even while it is being deposited. They lay their eggs uickly, and most leave within the first minute. The eggs are laid singly or in mall groups on the sides of the pat or on grass or on the ground near the pat.

The differences between the species in the structure of the respiratory ystem may be conveniently summarized in the form of a key, as follows:

1. Aeropyles connecting outer and inner meshwork layers of shell not more than 2 to 3 times as long as broad 2
— Aeropyles connecting outer and inner meshwork layers of shell more than 10 times as long as broad (figs. 8, B; 9, A, C) 6
2. With a large anterior respiratory horn which is about $\frac{1}{4}$ as long as egg. Outer layer of shell except median area between hatching lines and surface of respiratory horn a continuous sheet . *Musca* (*Eumusca*) *autumnalis* Deg.
— Without a respiratory horn. Outer layer of shell an open network (fig. 1) 3
3. Egg 4 mm long. Aeropyles about as long as broad. Inner meshwork layer of shell (fig. 7, B) 7 or more times as thick as outer meshwork layer *Mesembrina meridiana* L.
— Egg less than 2·5 mm long. Aeropyles about twice as long as broad. Inner meshwork layer of shell never as much as twice as thick as outer, usually thinner than outer 4
4. Outer meshwork layer of posterior pole with a system of wide canals arranged in an hexagonal pattern; canals of outer layer directly connected to canals of inner meshwork layer (fig. 4, C) *Orthellia caesarion* Meig., *Morellia hortorum* Fall.
— Outer meshwork layer of posterior pole without wide canals . . . 5

5. Outer surface of middle layer of shell not produced to form an hexagonal pattern of low ridges. Median area with struts of outer meshwork layer that abut upon hatching line mostly simple, not branched before their apices *Musca domestica* L.
— Outer surface of middle layer of shell produced to form an hexagonal pattern of low ridges directly above the hexagonal pattern of canals of inner meshwork layer. Median area with struts of outer meshwork layer that abut upon hatching line much branched before their apices (fig. 6, C) *Dasyphora cyanella* Meig., *Pyrellia cyanicolor* Zett.
6. Egg 2·2–2·3 mm long. Outer meshwork layer of shell distinctly thicker than inner (fig. 8, B). Aeropyles more or less equal in width and less than 40 times as long as broad (fig. 8, B) . . . . . . *Polietes lardaria* F.
— Egg 1·0–1·6 mm long. Outer meshwork layer of shell distinctly thinner than inner (fig. 9, A, C). Aeropyles more or less equal in width (fig. 9, C) or of two sizes (fig. 9, A); narrow aeropyles more than 50 times as long as broad. Stomoxidiini 7
7. Some aeropyles about 5 times as broad as others (fig. 9, A); broad aeropyles always above canals that form hexagonal pattern in meshwork of inner layer (fig. 9, A, B) . . . . . . *Haematobia stimulans* Meig.
— All aeropyles more or less equal in width (fig. 9, C) 8
8. Area between hatching lines with outer surface of middle layer of chorion not distinctly raised above canals that form the hexagonal pattern in inner meshwork layer . . . . *Stomoxys calcitrans* L.
— Area between hatching lines with most of outer surface of middle layer of chorion distinctly raised above canals that form the hexagonal pattern in inner meshwork layer so that there is a very distinct hexagonal pattern of low ridges *Lyperosia irritans* L.

My best thanks are due to Mr. E. A. Fonseca and Dr. J. C. Hartley for assistance in collecting the eggs, to Mr. A. A. Green for eggs of the common house-fly, and to Mrs. Joyce Ablett for technical assistance.

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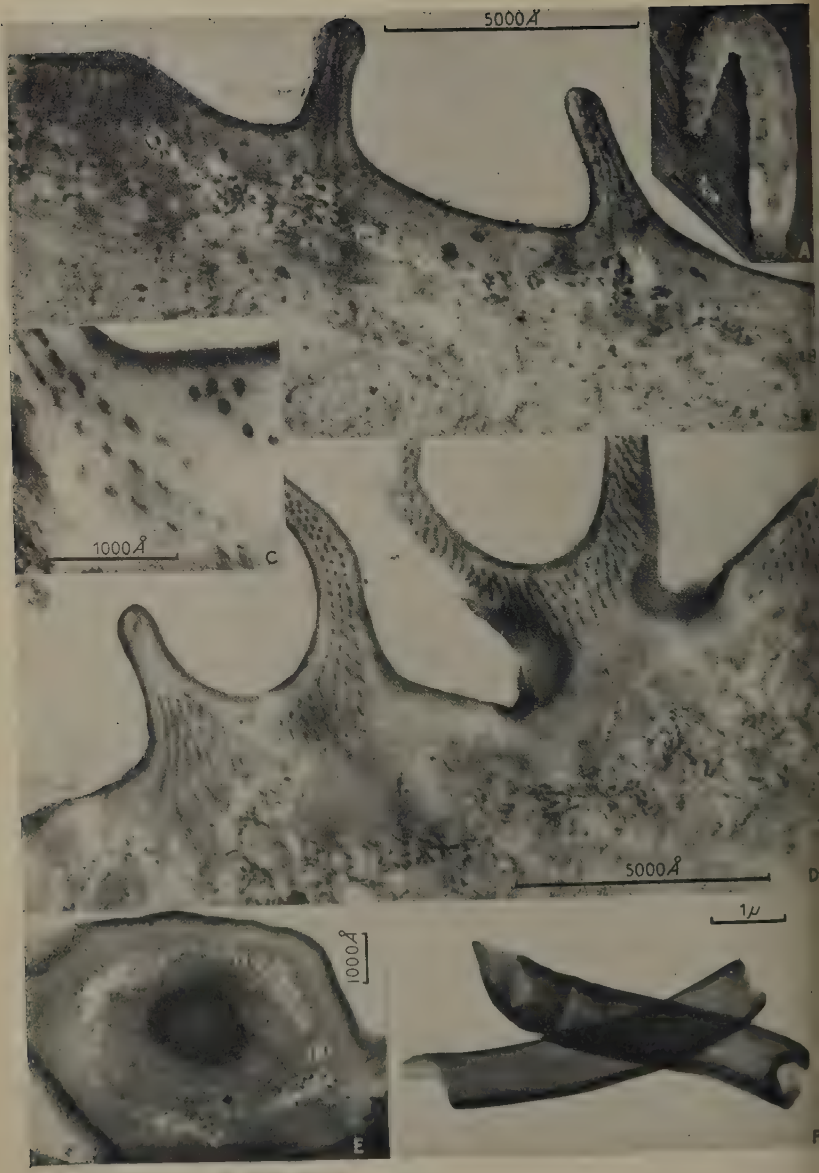

FIG. 1

M. LOCKE

The Cuticle and Wax Secretion in *Calpodes ethlius* (Lepidoptera, Hesperidae)

By M. LOCKE

rom the Department of Zoology, University of Cambridge; on leave from the Department of Zoology, University College of the West Indies, Jamaica)

With three plates (figs. 1 to 3)

SUMMARY

Larvae of *Calpodes ethlius* have an area of integument specialized for the secretion f long hollow filaments of wax. The epicuticle in this region has numerous crateriaped tubercles about 0·5 μ in diameter, from the rims of which the filaments are ecreted. Rows of tubes 60 Å in diameter emerge round the rim of each crater. The ax is supposed to traverse these tubes and they have been termed 'wax canals'. On ie inside the wax canals ramify irregularly. Tubes that appear to be similar occur sewhere in the epicuticle, where they may be concerned with normal wax secretion. he epicuticle consists of cement, a cuticulin layer 60 to 100 Å thick, and an inner ense layer of variable thickness. Microfibres are described in the endocuticle.

INTRODUCTION

ARVAE of many but not all of the family Hesperidae secrete paired patches of white powdery wax on the underside of the abdomen of segments 7 id 8 shortly before pupation. These are the 'glandes cirières' of Grassé (1951). hey have been known since early in the last century (Stainton, 1857), but ery little studied. In the *Canna* and arrowroot leaf roller, *Calpodes ethlius* toll, the glands are large and conspicuous (fig. 1, A) and provide convenient iaterial for a study of the problem of how an insect wax is secreted through the iticle. According to my own observations (Locke, 1959), the wax is syniesized in the epicuticle from water-soluble precursors, which can diffuse cross the endocuticle from the epidermis. Part of the evidence for this is ased on the absence of pore canals, which I failed to detect with the light icroscope in the endocuticle below the wax. An electron-microscope study as begun to check this observation, for it might be that the wax is transorted in pore canals below the resolution of the light microscope. A secondary object was to study the fine structure of the epicuticle itself, for if it is the

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FIG. 1 (plate). A, mature larva of *Calpodes ethlius* from the underside, showing the paired tches of white powdery wax at the posterior end.

B, vertical section through one of the crater-shaped tubercles which secrete the wax. lectron micrograph.)

C, a group of wax canals cut obliquely and a group cut almost transversely. (Electron icrograph.)

D, a pair of crater-shaped tubercles cut obliquely showing the wax canals passing into the bule layer. (Electron micrograph.)

E, transverse section through the base of a crater. Note the wax canals emerging into the ear space below the dense layer. (Electron micrograph.)

F, two fragments of a secreted hollow wax filament. (Electron micrograph.)

uarterly Journal of Microscopical Science, Vol. 101, part 3, pp. 333–8, Sept. 1960.]

site of esterases and wax synthesis it might be expected to show some structural modification adapted to this function. As an aid to this the structure of the cuticle in the wax-secreting area has been compared with the cuticle from other parts of the integument with normal wax secretion. The endocuticle was a third object of study, for if the lamellae are composed of dense sheets of chitin/protein they might provide a formidable barrier to the transport of large molecules.

Material and Methods

Larvae were fixed in ice-cold 2% osmium tetroxide solution at pH 7·2, and embedded in a mixture of equal volumes of methyl and butyl methacrylate. The cuticle is harder than methacrylate and tends to tear away along the outer edge but fairly good sections could still be cut at 200 to 300 Å on an A. Huxley type ultramicrotome. A number of stains were used on the sections but lead hydroxide prepared according to Watson (1958) revealed most detail in the cuticle. Many structures such as the microfibres in the endocuticle are only dimly discernible without staining, but after lead hydroxide show greatly enhanced contrast. Photographs were taken at magnifications up to 120,000 on a Metropolitan Vickers E.M. 6 or a Siemens Elmiskop 1 electron microscope.

The Cuticle over the Wax-secreting Area, and the Wax

General remarks

In fresh material with the phase-contrast microscope and in routinely fixed and stained cuticle only two regions can be distinguished with certainty—a lamellated endocuticle without pore canals and an epicuticle appearing in sections as a thin refractile line. The epicuticle has a curious pattern. There are polygonal areas with raised edges approximately equal to the areas of the epidermal cells, and scattered all over—several hundred to each polygon—are minute tubercles with depressed centres like miniature craters. These are at the limit of resolution and in surface view appear as dark circles under the highest powers of the phase-contrast microscope (fig. 2, A). The wax is secreted as fine filaments less than 1 μ in diameter from each of these craters. The filaments may grow to as long as 500 μ before being removed and wiped round the inside of the cocoon by the caterpillar.

The endocuticle

No trace of pore canals can be discerned with the electron microscope. The lamellae are variably spaced from 0·1 μ to 1 μ apart, and under high resolution are seen to be made up, at least in part, of microfibres not more than 25 Å in diameter. The fibres tend to be densely packed parallel to one another in

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Fig. 2 (plate). A, surface view of the cuticle which secretes the wax. The crater-shaped tubercles show up as dark circles. Phase contrast.

B, section tangential to the surface of the cuticle, cutting the craters in transverse section. Note the regular array of wax canals. (Electron micrograph.)

C, small part of B at a higher magnification.

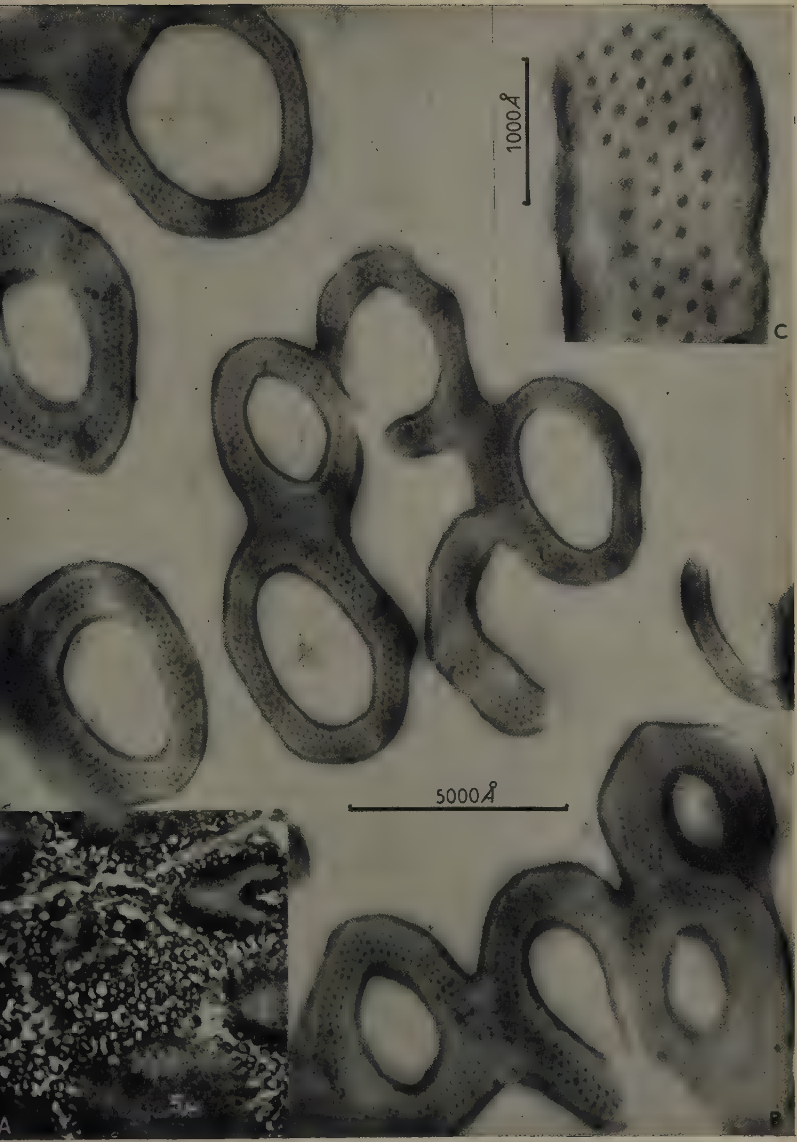

Fig. 2

M. LOCKE

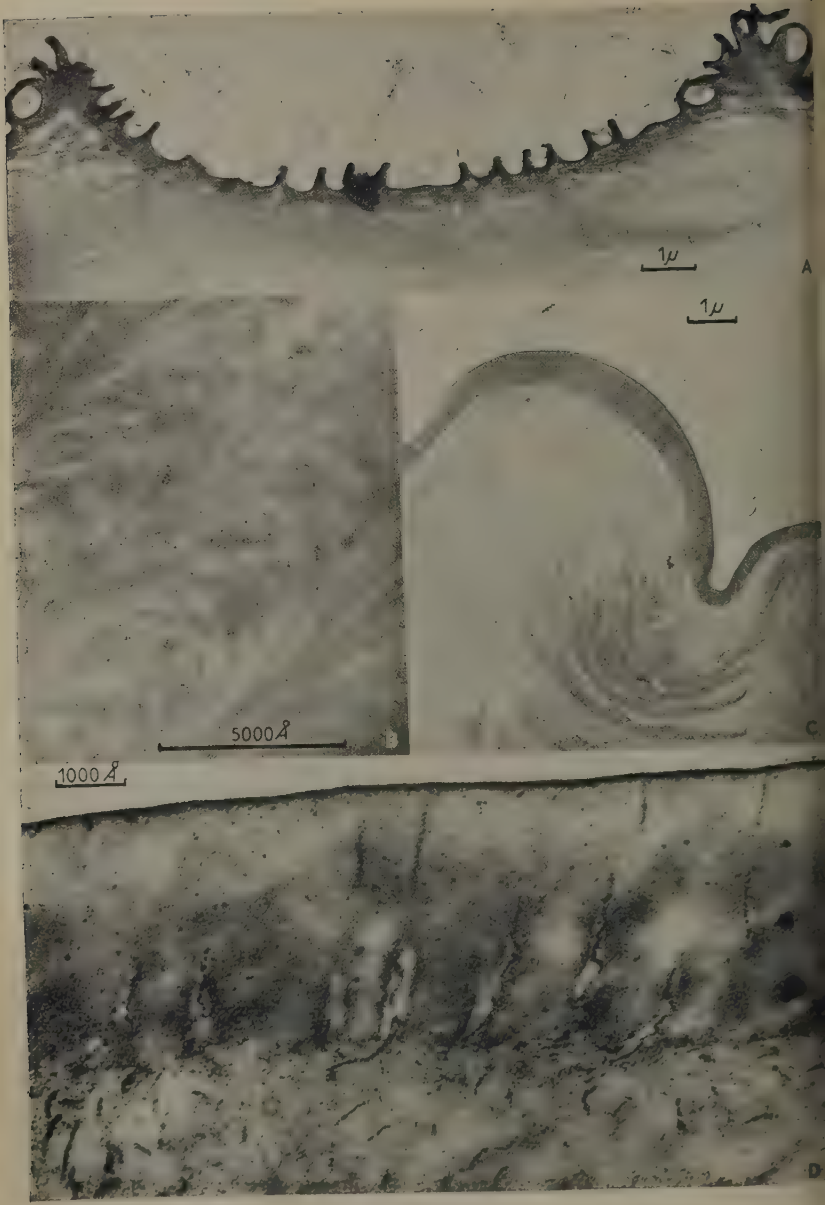

FIG. 3

M. LOCKE

e centre of a lamella, but they fan out at angles up to 90° into the less well-dered region between lamellae (figs. 3, A, B). When the endocuticle is being id down, the epithelium appears to be secreting material which only takes the rm of lamellae about 2 μ away from the cell membrane.

he epicuticle

Cement. Even before wax secretion the cement rarely survives embedding a distinct layer but fragments away from the surface. This is not surprisg if the cement is a shellac-like substance, as Beament (1955) suggests. In g. 3, A it appears as loose strands some way from the surface. During wax cretion it is pushed right away from the surface.

Cuticulin. The term 'cuticulin' has been retained for the very dense, heavily aining outer layer, about 60 to 100 Å thick, which follows even the finest ulpturing of the cuticle. It is probably not just a region of the layer below, condarily differentiated by a chemical change at the surface, for it somenes appears on its own above occasional granules in the dense layer below.

Dense layer. Below the cuticulin and including all the wall of the wax-secretg craters is a dense, homogeneous layer about 1,000 Å thick (figs. 1, B–E; B, C). Its inner surface is less regular and may extend in small islands.

Wax canals and convoluted tubules. Emerging round the periphery of each ater there are 3 or 4 rows of tubes about 60 Å in diameter (fig. 1, B, D). The bes do not always appear hollow (fig. 1, C) and their contrast with the rest the cuticle is probably due to staining of the material which they contain. ithout lead staining they are barely distinguishable. The arrangement of the ws is strikingly regular, each tube being equidistant (about 160 Å) from neighbours (fig. 2, B, C). On the inside the tubes ramify in an irregular way ter they emerge from the dense layer, and in this region the cuticle round em appears less dense. The less-dense areas are particularly obvious at the se of each crater, where they extend into the dense layer as the tubes emerge g. 1, E). There is a matrix of microfibres without any preferred orientation parating the lamellae of the endocuticle from the region where the tubes mify.

The hollow wax cylinders. The wax filaments seen with the light microope have a melting-point of 81 or 82° C and they can be viewed in the ectron microscope without melting. Under this higher resolution they are en to be hollow cylinders with the same diameter as the apices of the aters which secrete them (fig. 1, F). The wax follows exactly the distribution

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FIG. 3 (plate). A, Transverse section of the cuticle where the wax secretion takes place. ote the absence of pore canals and the fragmented cement layer. (Electron micrograph.)

B, section at right angles to the plane of the lamellae in the endocuticle. Three lamellae are ible crossing the field obliquely. (Electron micrograph.)

C, vertical section through a tubercle on a tergite. Note the gaps in the epicuticle which ompany the wax canals. (Electron micrograph.)

D, the epicuticle and wax canals from a tergite at higher magnification than C. (Electron crograph.)

of tubes in the walls of the crater, and this provides strong circumstantial evidence that it is secreted through them. The tubes have therefore been termed 'wax canals'. For the same reason it seems probable that the wax canals traverse not only the dense layer but also the cuticulin layer and they have been drawn thus in fig. 4, although they cannot with certainty be seen to do so. Fig. 4 presents in diagrammatic form the chief findings concerning the structure responsible for the secretion of a single hollow wax cylinder.

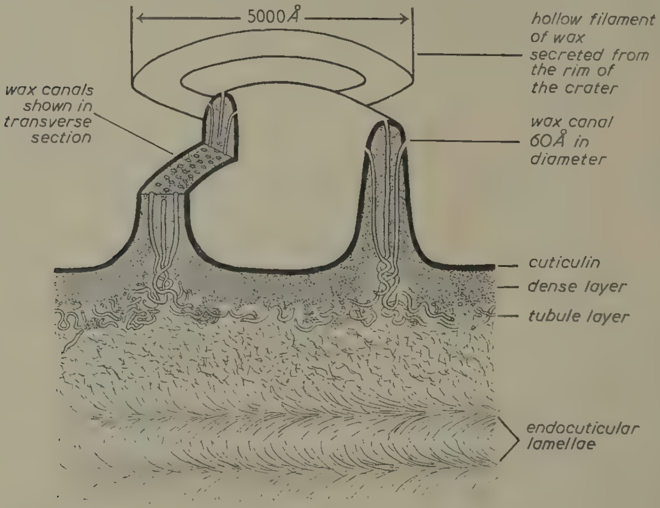
5000Å
hollow filament of wax secreted from the rim of the crater
wax canals shown in transverse section
wax canal 60Å in diameter
cuticulin
dense layer
tubule layer
endocuticular lamellae

FIG. 4. Diagram of the most probable structure of the cuticle which secretes the hollow wax filaments.

CUTICLE FROM OTHER PARTS OF THE CATERPILLAR

Over most of the sternites and tergites the cuticle is thrown into rounded tubercles similar to those described by Way (1950) and Takahashi (1956). With the electron microscope these are seen to have basically the same components as the wax-secreting cuticle (figs. 3, C, D). The cement layer is irregularly preserved. The cuticulin layer is of approximately the same thickness as in the wax-secreting cuticle, but the dense layer is everywhere much thicker. There are a few tubes penetrating the dense layer, having the same dimensions as the wax canals, but they are irregularly and sparsely scattered. For about two-thirds of their journey through the dense layer they are accompanied by a finger-like projection of less dense material from the inside. The tubes themselves ramify little when they emerge, but pursue a much straighter course for a short distance into the non-laminated endocuticle. The endocuticle does not differ from that described already.

The epicuticle has also been studied on the tracheae. It has no wax canals nd is similar to that described in *Rhodnius* and other insects, consisting of wo layers, cuticulin and the dense layer (Locke, 1957).

The chief features of the tergal cuticle are summarized in fig. 5.

Discussion

Although the epicuticle is known to contain waxes (Wigglesworth, 1957), t is perhaps not surprising from its dense appearance under the electron nicroscope that pores through it should be necessary for the movement of vax. But if the wax canals were only necessary to provide unimpeded move- nent through the epicuticle, they might be expected to terminate or even unnel out on the inner surface. However, the wax canals are more than this, or the straight course of the canal through the epicuticle is only a half or a hird of the total length of the tube. The convoluted course is improbable if he tube is only a vehicle for wax-transport from the inside to the outside of he epicuticle. If, as has been suggested, the wax is synthesized in this region, would seem plausible to suppose that the site of synthesis should be as close o the site of deposition as possible. This being so, the walls of the convoluted ube have an obvious claim as a seat for this synthesis. Two other details sup- ort this hypothesis. The cuticle round the convoluted tubes is less dense and ooks as though it might provide free access to them, and in the tergal cuticle here is an apparently empty sleeve for the tube for over half its passage hrough the epicuticle.

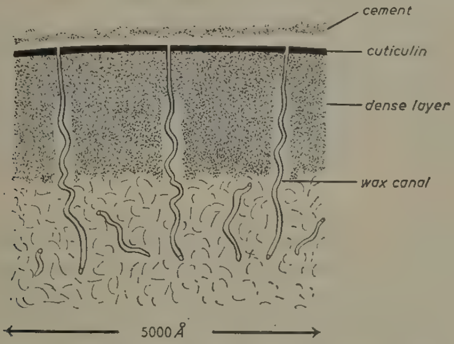

Fig. 5. Diagram of the most probable structure of the epicuticle over most of the body.

If wax canals should prove a common feature in the wax secretion of in- ects, they may play a significant part in the permeability of the cuticle, parti- ularly to non-polar substances, including many insecticides. There is some hysiological evidence favouring this. Holdgate (1956), for example, suggests at water loss/temperature curves may be explained by diffusion through inholes.

According to Watson (1958) lead hydroxide stains many cellular con- tituents. A possible rationale for its staining action in the epicuticle would be the formation of lead soaps from fatty acids, if the fatty acids were not entirely leached out during embedding.

The double nature of the epicuticle (excluding the cement) is now well documented for a number of insects (*Sarcophaga*, Dennell, 1946; *Calliphora*, Wolfe, 1954; *Periplaneta*, Dennell and Malek, 1955; *Apis*, Richards, 1952; *Bombyx*, Ito, 1954; *Rhodnius*, Locke, 1957, 1958). For convenience in this paper the outer layer has been referred to as cuticulin, although the term as introduced applied to both layers. Its characteristic feature in *Calpodes* as in *Rhodnius* is its extreme thinness and uniformity. The thicker layer within has been referred to with a non-committal term, the dense layer. No more specific name is useful until its chemical nature has been established. In tracheae it contains chitin but this may not be true in general.

According to Richards (1958, 1959) microfibres have not been found in cuticle, nor from his cross-grid model of chitin/protein structure, based on his work on the optical properties of isolated chitin and anthropodin, does he expect to find them. Fig. 3, B shows that such fibres exist and their orientation may go some way to explain his results. The effect of the orientation on the integument may be to allow the stretching which occurs during an instar and it might also impart some elasticity.

I greatly appreciated the hospitality of Professor V. B. Wigglesworth during my 6 months' stay in his laboratory. I am very grateful also to Dr. V. E. Cosslett for the electron-microscope facilities provided in the electron-microscope department of the Cavendish Laboratory. I particularly thank Mrs. A. Cosslett for her helpful advice and the use of her equipment for the preparation of ultra-thin sections. My thanks are also due to Mr. T. Morley and Mr. R. Horne for taking the electron micrographs. Lastly, I am happy to acknowledge my debt to Dr. J. W. L. Beament's encouragement.

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Contractility and Hydroplasmic Movements in the Hydroid *Clytia johnstoni*

By L. J. HALE

From the Department of Zoology, University of Edinburgh)

With one plate (fig. 1)

SUMMARY

The investigation is based on observations of the living animal and on time-lapse ilms, and is supplemented by histological preparations. The material was cultured in he laboratory.

A short region just behind the growing tip of a stolon is contractile, pulsating every 4 or 5 min but occasionally at longer intervals. This causes the hydroplasm (contents of coelenteron) to ebb and flow in the coelenteron not only directly, but also by pressure transmitted by the fluid between the coenosarc and perisarc. There is evidence that where a stolon has more than one contractile region, they beat independently. The contractile cells do not possess muscle-tails, and contractile fibres have not been detected by light microscopy.

The hydranth stomach also has slow contractions which are peristaltic towards the stalk. They usually occur at irregular intervals but are occasionally regular. They cause movement of the hydroplasm into and out of the hydranth. There is evidence that hydranths are stimulated to expand and contract by arrival of hydroplasm pressed there by another contractile region of the colony. Longitudinal muscle-tails are present on the ectoderm cells of the stomach and tentacles, but these are probably concerned with retraction of the hydranth into its hydrotheca.

Although the endodermal flagella beat actively when the hydroplasm is in motion, they are not the cause of hydroplasmic flow.

The possible mechanism of these slow contractions is discussed in relation to contractility in other coelenterates.

INTRODUCTION

A WELL-KNOWN feature of the colonial hydroid is the slow ebb and flow of the hydroplasm; a movement first seen by Agassiz (1862) in the hydranths of a number of hydroids. Hammett and Padis (1935) first noted expansions and contractions of the stomach in *Obelia*, and later Berrill (1949) recorded expansions and contractions in a region just behind the growing point of a stolon in the same animal; in both cases these were related to the movement of the hydroplasm.

The hydroplasm is well known as being important in the transfer of food materials from the hydranths to all parts of the colony. It also contains cells, at least partly derived from regressing parts of the colony (Huxley and de Beer, 1923). It has been found that the cells derived from a regressing part of the colony are probably normally used again for the growth of other parts of the colony, and are transported by the hydroplasm. The hydroplasm thus plays an important ancillary part in the growth of the animal. Its mode of

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movement in a colony is reported here. The observations on growth will be recorded in another paper.

Clytia johnstoni Alder was chosen for this study because of its simplicity of morphological structure, the relatively large size of its cells, and its transparency which enabled the living animal to be studied with ease. It has long stolons (fig. 1, A) which occasionally branch but do not anastomose. The hydranths are normally borne singly on their stalks, and reproductive zooids when present are found either on the stolon or part of the way up the hydranth stalks. In the latter two respects it differs from *Obelia*, which it otherwise closely resembles.

It will be shown here that the hydranth stomach and a region near the growing point of a stolon are indeed the only contractile regions in a *Clytia* colony concerned with hydroplasmic movements; the stolonic region is the more important. The contractile cells do not possess the muscle-tails characteristically associated with coelenterate contractility.

MATERIALS AND METHODS

Colonies of *Clytia* were cultured in the laboratory. Material collected on the shore, and growing upon red algae, was attached to glass plates, and kept in a rather fast stream of sea-water (Hale, 1957). They were fed daily (twice daily for fast growth) on newly hatched nauplii of *Artemia salina*. Cultures were kept in darkness to inhibit the growth of diatoms and other algae which would otherwise hinder observation of the hydroid. The water temperature was controlled at 16° to 18° C.; evaporation losses were made good with rain-water; the water was occasionally changed (4 to 8 weeks). Under these conditions the colonies grow over the glass plates from the explant attached to its algal substratum; the latter is then removed. Subcultures are easily made.

The first stolons to grow are firmly attached to the plate, but later 'free' stolons are produced as well; these also branch and produce hydranths. These unattached parts of the colonies form the most convenient source of material for observation. As stolons change spontaneously from the attached to the free state and vice versa, it is concluded that, as far as the present work is concerned, there is no difference between them (their similarity was noted by Berrill, 1949).

A small perspex cell was used for observation of small colonies and parts of colonies with the microscope and for photography; inlet and outlet tubes were fitted to carry a continuous flow of sea-water through it. *Clytia* lives quite normally in such a chamber, for 2 weeks or more without food. Observations considered in this paper were made on animals kept in the chamber for only a day or two.

The results recorded here are based mainly on observation of the living

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FIG. 1 (plate). A, *Clytia johnstoni* Alder; a colony growing on the red alga *Polysiphonia*. B, phase-contrast photomicrographs of the growing end of a stolon. On the left the contractile region is expanded and on the right it is contracted (ectoderm pale, endoderm dark).

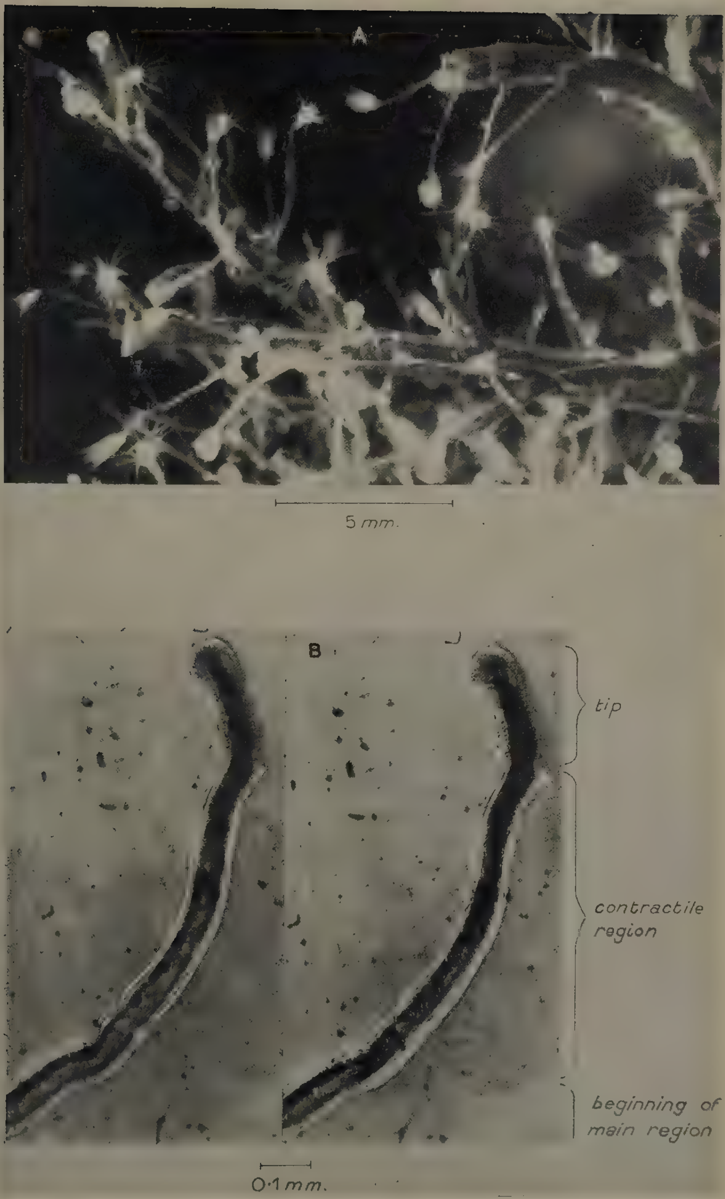
A
5 mm.
B
tip
contractile region
beginning of main region
0·1 mm.

FIG. 1
L. J. HALE

animal and on time-lapse films taken of it on 16-mm Micro Neg Pan film (Ilford). Very low magnification (×1 to ×2) was required to record contractions in whole colonies, and the problem of evenly illuminating such an object was solved by focusing the enlarged image of a strip filament lamp by a condensing lens on to a piece of opal glass immediately beneath the observation cell. Films were also taken with 8-mm and 16-mm phase-contrast objectives. Exposures were made mostly at 5-sec. intervals, but some were taken every 20 sec.

To determine the relationship between contractions in various parts of the colony, the low-power films were projected a number of times and at each showing the incidence of pulsations in one contractile region was timed.

Observations on the living animal were supplemented by histological preparations. Material was fixed in 5% formalin in sea-water, Bouin's fluid, and Heidenhain's Susa, and was mounted whole or sectioned. It was stained with a variety of dyes, but especially by Mallory's method and Heidenhain's iron haematoxylin; the latter method was by far the most revealing. Macerated preparations were also made with Goodrich's iodized concentrated boric acid solution in sea-water (Goodrich, 1942); they required about a week in the solution for successful preparations. Phase contrast and polarized light were used to observe the isolated cells.

Gonangia were omitted from the investigation; they are temporary structures and cannot therefore be of importance in this study.

RESULTS

Stolon

Three regions may be distinguished in a stolon (fig. 1, B). In the greater part of a stolon, which may be several cm in length, the coenosarc is separated from the stiff perisarc tube by a fluid-filled space and occasional ectodermal struts. The lumen of the coenosarc is always open.

The growing end of a stolon is distinguished by two short regions. At the tip (about 0·5 mm) perisarc and coenosarc adhere, and the lumen is occluded for most of the time. Next follows the contractile region (about 1·0 mm), which is similar to the main part of the stolon except that when the tube is expanded the space between the perisarc and coenosarc disappears, and when it is contracted the lumen is almost occluded. The end of a stolon which is not growing does not possess these two regions.

Time-lapse films clearly reveal the contractile region of a stolon, and it undoubtedly corresponds to that seen by Berrill (1949) in *Obelia*. Its length varies from 0·3 to 1·8 mm; in well-established stolons it is usually but not always longer, and in a regenerating stolon the region is shorter.

The resting state is when the tube is contracted; it is the longer period of the cycle, and is the state of the tube when anaesthetized. Expansion starts somewhere in the middle of the contractile region, its precise position being variable, and then spreads towards both ends (fig. 2); it is completed in about

½ min. When expanded the space between coenosarc and perisarc is obliterated, the fluid having been pressed away from the growing end. Hydroplasm streams into the contractile region.

Contraction is slower, lasting 1 to 2 min. It takes place more rapidly near the end of the contractile region distal to the stolon tip, and spreads proximally. Thus hydroplasm not only returns down the stolon, but is also pressed into the tip. The extreme end bulges out to accommodate this pressure; the perisarc is soft here being newly secreted. As the contraction dies away the fluid in the tip runs back into the main part of the stolon. Then ensues a quiescent period of some 2 or 3 min.

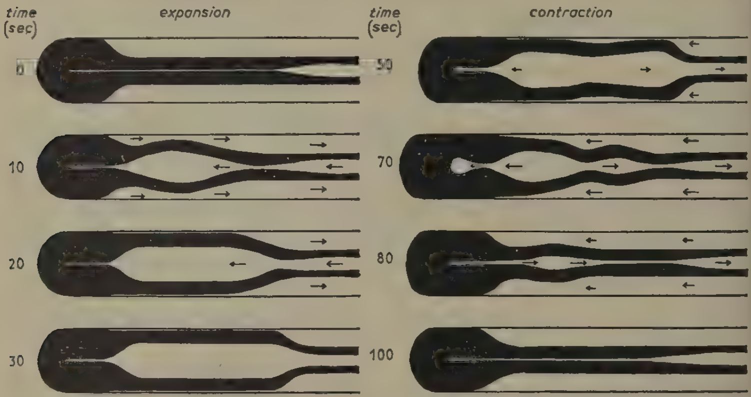

FIG. 2. Changes in the shape of the contractile region of a stolon, reconstructed from a film. The part of the stolon shown is 1 mm in length; its diameter (80 μ) is exaggerated about 2½ times. Arrows indicate fluid movements.

In all of the 16 films taken of colonies or parts of colonies with a growing stolon the contractile region is seen to pulsate rhythmically (fig. 3). It continues to pulsate when all hydranths have been removed (fig. 3, E, J, Q), which shows that it possesses contractility, and is not being passively dilated by another contractile region in the colony.

Measurements made from the films (fig. 3) show that in 13 of the 16 colonies pulsations occur on the average every 4½ min (18±1° C), the range being from just under 4 min to about 5½ min; it is often surprisingly constant in any one stolon. In the other 3 cases the pulsations took place every 7 or 8 min. Why these should beat more slowly has not been discovered; it is not due to the size of the contractile region, to the size of the colony, or to its constitution (number of hydranths, &c.). Although temperature changes would be expected to change the frequency of beat, this is not the reason in these cases.

Where a stolon has a contractile region at both ends, there is evidence that they contract independently; this can be seen in colony F (fig. 3).

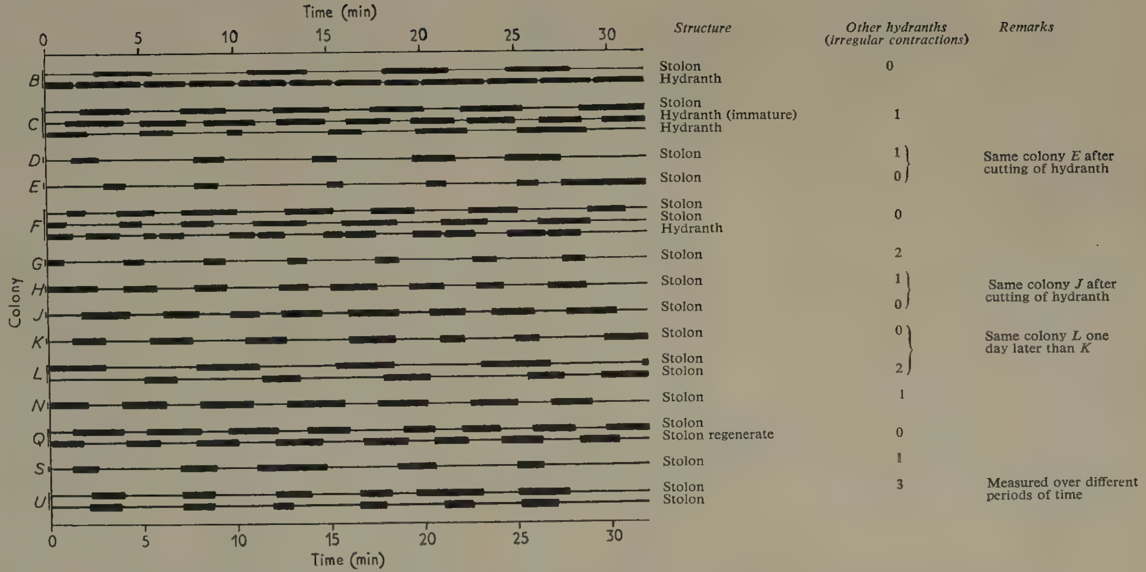

FIG. 3. Times when regularly pulsating parts of colonies are expanded (thick line) and contracted (thin line).

The cells in the contractile region of a stolon (fig. 4) never possess the muscle-tails characteristically associated with coelenterate contractility. With phase contrast such contractile fibrils produce high contrast and are easily detectable; no such fibrils could be found in either ectoderm or endoderm cells. No birefringence is seen in ectoderm cells with polarized light. Endoderm cells contain many very strongly birefringent spherules (probably lipid), which would make observation of fibrils with less birefringence difficult. The bases of these cells have fewer of these spherules but no birefringence is seen there. No fibrillar structure can be found in fixed and stained preparations. It may be concluded that it is unlikely that the contractile region of the stolon possesses fibres large enough to be detected by visible light.

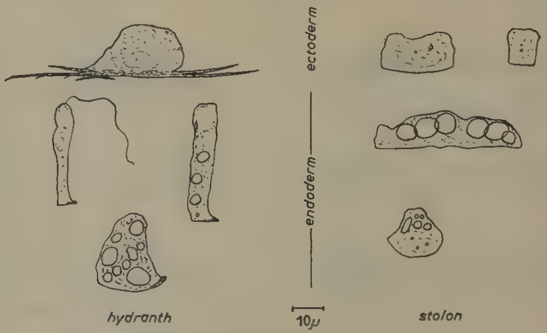

FIG. 4. Cells isolated by Goodrich's method. Contractile fibrils are found in ectoderm cells of the stomach and tentacles (longitudinally arranged), but nowhere else in a colony.

The ectodermal struts connecting the coenosarc with the perisarc could cause expansion of the stolon if their constituent cells contracted. Each strut consists of one or two small, elongate ectodermal cells. They are usually found in the short contractile regions of a stolon, but they are sometimes sparse or even absent altogether. Most are arranged roughly perpendicular to coenosarc and perisarc, but occasionally one can be found at a considerable angle. If one of these contracted some distortion of the coenosarc would take place, but this has not been detected. As the struts are occasionally absent, there is no indication that they are any more than passive supports.

The extreme tip of the growing stolon also shows pulsations, but it has been shown above that they are almost certainly due to passive dilatations caused by the contraction of the region behind. The end of a stolon which is not growing is not contractile. Frequently the cut end of a stolon begins to regenerate. Such a newly-formed outgrowth also pulsates a little, but again they are almost certainly passive dilatations. In one experiment a short length of stolon was taken and the hydranths and their stalks removed. At one end was a normal contractile region whose contraction caused the other regenerating end to enlarge, and whose expansion resulted in the collapse of the regenerate, as the hydroplasm was moved alternately away from and towards the contractile region (fig. 3, Q).

The main length of the stolon is almost without movement during the flow of the hydroplasm, but a very slight change in its diameter can sometimes be detected. Since the perisarc is a hard, inflexible, sealed tube, activity of the contractile region of the stolon must cause movements not only of the hydroplasm, but also of the fluid lying between the coenosarc and the perisarc (fig. 2). It is these movements which cause the slight alterations in the diameter of the coenosarc. This double action pumping system will be recognized as an efficient one.

The action of the flagella of the endoderm cells may also contribute to hydroplasmic movement, as they always beat energetically when the coelenteric fluid is in motion. They do not beat with metachronal rhythm, but they might all be beating in the same direction but randomly out of phase with one another. They stir up the coelenteric fluid so that the contained cells and particles jiggle about as they are moved along. Treatment of colonies with 1 : 2,000 chloretone soon causes a complete cessation of pulsations in the tolon, but it appears to have no inhibitory effect on the flagella. In such anaesthetized animals no flagellar beat is possible in the tip nor in the conractile region of a stolon, as the lumen is occluded. In the main part of the tolon flagella beat more or less continuously in some parts, spasmodically n others, and with an activity seemingly no different from that which occurs n the normal animal. This activity stirs up the coelenteric contents, but nothing except random movements of cells and particles is seen. The activity of the flagella thus do not contribute to hydroplasmic motion.

Hydranth

The hydranth possesses 4 quite distinct kinds of contractility. One or a few of the tentacles will occasionally exhibit a slight waving motion, but they often remain for long periods (up to $\frac{1}{2}$ h or more) without moving. They move when prey is captured and swallowed, but this is again a slow movement and is usually confined to the tentacles concerned in the capture.

On capturing food the mouth opens and closes actively and repeatedly. The whole buccal region moves as well, tending to lean towards the side where the food lies. These movements are not confined to times when food has been captured; they have been frequently seen and also recorded on films n conditions where no food organisms were present.

A third type of contractility is the complete withdrawal of the hydranth within its hydrotheca. This will take place not only as a result of a mechanical stimulus, but also without any apparent stimulus. The contraction is the only rapid one found in the animal and takes place in a fraction of a second. In his withdrawal the tentacles are considerably shortened, and the stomach region either becomes short and wide or remains thin and becomes S-shaped. Expansion is very much slower, taking about 10 to 20 sec. During the contraction there is no movement of the hydroplasm out of the hydranth into its stalk, so that the normally open junction between hydranth and stalk must close at this time. Unstimulated retractions normally occur infrequently, usually once in periods of 20 min or more, but occasionally more often. Hydranths isolated with their stalks retract frequently, every $\frac{1}{2}$ to 2 min.

The fourth type of contractility is exhibited by the stomach only (first noted in *Obelia* by Hammett and Padis, 1935). It is very slow, its speed being about the same as that of the contractile region of the stolon. In time-lapse films the contraction is seen to be always peristaltic, waves of expansion and contraction passing slowly from the distal end of the stomach towards the stalk. The occurrence of an expansion and contraction is usually quite irregular, and varies from hydranth to hydranth even in the same colony. A pulse may or may not be immediately followed without pause by one or more further contractions, and there are usually quiescent periods at variable intervals (some exceptions to this are considered below).

Isolated hydranths show a more or less continuous peristaltic activity of the stomach. This shows that the stomach is actively contractile and is not being dilated by another contractile region in the colony.

Surges of hydroplasm accompany activity of the stomach. On expansion the fluid and its contents flow from the stolon through the stalk into the stomach, and they return in the same way on contraction of the stomach.

The hydranth stomach is in direct connexion with the surrounding sea-water, so that its expansions and contractions cannot have the double pumping action possessed by the stolonic contractile region. It must therefore be less efficient in moving the hydroplasm.

Of 20 film records of fully developed hydranths and of hydranths in the later stages of development, 16 possessed irregular contractility and the other 4 showed some regularity in their contractions. One hydranth was peculiar (fig. 3, B) in that most of the time the stomach was swollen, but it contracted regularly about every 165 sec; there was no clear relationship between its contractions and those in the stolon. A second colony (fig. 3, C) consisted of a length of stolon with 3 hydranths at intervals: near the growing end was a hydranth whose differentiation was almost complete, and whose stomach pulsated regularly but without any obvious relation to the pulsations of the stolon; the stomach of a second hydranth (fully developed) pulsated alternately with the stolon; and near the other end of the stolon was a third hydranth whose pulsations were quite irregular. The other regularly contracting hydranth was in a colony where the hydranth had a stolon growing out of its stalk (a rare situation); the hydranth pulsations alternated with those in the stolon (fig. 3, F).

In this last case, stolon and hydranth are abnormally close to one another, and hydroplasm pressed out of the stolon into the hydranth may have stimulated the latter to expand. Further support for such a suggestion comes from the fact that in 5 of the 6 periods of hydranth activity observed, two expansions and contractions followed one another without pause. Now the nature of stolon contraction is likely to give rise to a double movement of hydroplasm out of the stolon, the first directly out of it and the second by the escape of luid previously pressed into the tip. Movement of fluid through a longer length of coenosarc might be expected to merge these two movements of fluid into one, and perhaps this is the reason for the absence of any evidence of double contractions in other hydranths.

The evidence that contractility of the stomach is stimulated by the arrival of hydroplasm explains why a hydranth usually beats irregularly. The intervals between the arrivals of hydroplasm will depend on the lengths and arrangement of the various coenosarc tubules connecting it to other pulsating parts of the colony; these intervals will usually be irregular. Occasionally an arrangement will give rise to regular pulsations, and this is probably what is happening n colony C. Attempts to determine whether two hydranths would stimulate each other to contract when connected with a short length of coenosarc were nconclusive.

A hydranth branch usually originates in the contractile region of a stolon but occasionally elsewhere). For a time as the hydranth stalk grows away rom the substratum, the proximal part of it expands and contracts in unison with the adjacent stolon. This part of the stalk is actively contractile; if it were not then it would dilate when the stolon contracted. When the stalk is about half its final length its contractions die away, although the adjacent stolon may till be contracting.

When a hydranth stalk has grown to full length, the end swells into a bulb. Both the tip of the stalk and the bulb pulsate in a very similar way to a stolon ip; they are also being dilated by hydroplasmic pressure from the stolon. The bulb differentiates into a hydranth, and as the pulsations become restricted o the developing stomach, they also become peristaltic like those of the adult tomach.

Hammett and Padis (1935) and Hammett (1943) described (in *Obelia*) the treaming of hydroplasm as towards a growing hydranth, alternately towards nd away from a fully developed hydranth, away from a regressing hydranth, nd no current 'where the ground is being prepared for new growth initiaion'. My observations in *Clytia* show that there is an equal movement both o and from all parts of a colony; indeed it is difficult to see how the fluid ould move otherwise in a system of branched, blind-ending, and nonnastomosing tubules. Hammett (1943) also states that in *Obelia* surges and ecessions of hydroplasmic pressure are aided by 'peristaltic-like activity within the pedicel'. In *Clytia*, apart from dilatations of the tip, contractions an be seen during the early part of stalk development, but not after this stage; erhaps it is these contractions to which Hammett refers.

In the hydranths characteristic musculo-epithelial cells are present, and in macerated preparations they are seen to have 1 to 3 or occasionally more muscle-tails (fig. 4). Sections and whole mounts show that the muscle-tails re parts of ectodermal cells, and run longitudinally. They occur in the tomach and tentacles but, curiously, are absent from the buccal region. Macerations of the hydranths also reveal the details of the endoderm cells (fig. 4); they are typical in shape and in possessing flagella, but their expanded bases lack the contractile fibres expected. Under phase contrast one end of the base sometimes possesses a short region of greater contrast, but it has been impossible to detect this in whole mounts, sections, or the living animal; the position of such cells and the orientation of their bases remain obscure.

DISCUSSION

Rhythmic contractility is an almost universal feature of multicellular animals, especially associated with the movement of fluid such as blood and food undergoing digestion. In these two important cases the rates of transportation are likely to be related to the needs of the animal for oxygen and food materials, and the rate of transportation will be reflected in the frequency and amplitude of the pumping mechanisms involved. It will thus be expected that this contractility will be rather infrequent and of small amplitude in sluggish animals and especially in sedentary ones. Such is indeed the case. In *Arenicola* (Wells, 1950) the feeding cycle occurs about every 7 min, and the irrigation cycle at about 40 min intervals. In the sedentary ascidian *Phallusia* there is spontaneous activity of the siphons every 6 to 9 min (Hoyle, 1952). The actinian *Metridium* (Batham and Pantin, 1954) contracts spontaneously every 2 to 12 min, and *Calliactis* (Needler and Ross, 1958) every 10 to 15 min. And in the hydroid *Clytia* the coelenteric fluid with its contained food materials and cells, but probably of no importance as an oxygen carrier, moves as a result of rhythmic contractions every 4 to 7 min.

Present knowledge of the muscular elements of coelenterates has recently been reviewed by Robson (1957), and it is unnecessary to repeat it here. In hydroids work has been mainly confined to *Hydra*, and in this animal it is clear that the ectoderm and endoderm cells both possess contractile fibrils, which run longitudinally and circularly respectively. The ectodermal cells possess 1 to 3 or more muscle-tails, but the endoderm cells are somewhat different in form; the cell base is much less expanded and contains less distinct contractile fibrils. The endoderm fibrils are also less developed than the ectodermal ones in the tentacles of *Metridium senile* (Batham and Pantin, 1951). Holmes (1950), on the contrary, found that in the tentacles of *Hydra* circular fibres are the more prominent; this may be connected with the great extensibility of the tentacles.

The studies on *Clytia* show that the longitudinal muscle-tails are almost certainly concerned with the sudden retraction of the hydranth into its cup. Those in the tentacles take part in this contraction as well, and they are probably concerned in the bending of the tentacles. They could not be responsible for the slow, peristaltic movements of the stomach, even if circular contractility were present, as no longitudinal shortening takes place. But it is interesting to note that Batham and Pantin (1954) found that some muscle-cells in *Metridium* took part in both slow and fast contractions. It is certain that in *Clytia* the cells concerned in rhythmic contractions do not contain myofibrils.

The changes in shape of the hydranth stomach and in the contractile region f the stolon can only be explained by postulating that expansion of the tube s caused by radial contraction of the cells (i.e. in a direction perpendicular to he axis of the tube), and contraction of the tube by circular contraction of the ells (i.e. circumferentially). There are marked changes in the shapes of the ells during expansion and contraction, especially in the endoderm cells (fig.). The radial dimension of the endoderm cells decreases to about a third after xpansion, and their bases (adjacent to the mesogloea) decrease to about 60% f their expanded length after contraction of the tube.

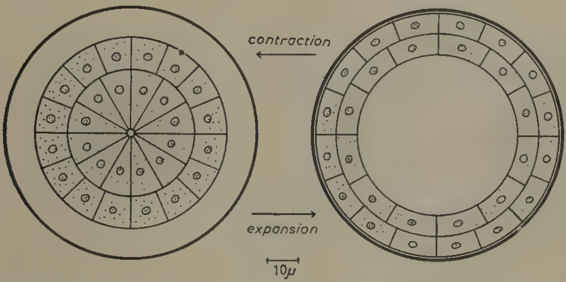

FIG. 5. Semi-diagrammatic reconstruction of transverse sections through the stolonic contractile region. During expansion and contraction of the tube the cells change their shape considerably, especially those in the endoderm.

Robson (1957) pointed out the great changes in shape occurring in the usculo-epithelial cells during expansion and contraction in *Metridium*. She lso drew attention to the fact that in these cells there are present not only ontractile fibrils running along the base of the cell into the muscle-tails, but lso vertical strands passing from the muscular part towards the free ('epithe-al') part. She was unable to decide whether the cell-bodies assisted in the hortening process, nor whether these vertical strands were contractile. Semal-an Ganson (1952) states that in the siphonophores *Apolemia invaria* and *orskalia ophiura* fibrils are found in the body as well as the base of musculo-pithelial cells.

The changes in cell shape in *Metridium* are thus basically similar to, but uch greater than those in *Clytia*. Also the suggested orientation of the con-actility in *Clytia* corresponds to the orientation of fibrils in the cells of *etridium*. It may be, therefore, that the essential features of the slow con-actility in *Clytia* are no different from those in other coelenterates, but they re very poorly developed.

It is a pleasure to acknowledge the hospitality and help of the Director and aff of the Stazione Zoologica, Naples, where preliminary observations were ade. I should also like to thank Professor M. M. Swann and Dr. R. A. Kille for kindly reading the manuscript, and Mr. D. Cremer for taking the photomicrographs and for assistance in the cinematography.

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Differences in the Blood-supply of Bone of Different Histological Types

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SUMMARY

'Laminar' bone, commonly found in artiodactyls and dinosaurs, is described. It consists of a series of bony laminae arranged like tree-rings round the marrow cavity, each lamina being between 0·1 and 0·2 mm thick. Sandwiched between each lamina and the next is an effectively two-dimensional network of blood-vessels. Laminar bone grows in a series of spurts; a sheet of woven-fibred bone is formed very quickly, not actually on the pre-existing sub-periosteal surface, but separated from it by a space. The new bone is held clear of the original surface by an occasional bridge of bone. The space so formed is filled in slowly by parallel-fibred lamellar bone, and while this is going on more woven bone, with spaces, may be formed by the periosteum.

The vascularization of laminar bone on the one hand and bone composed of haversian systems on the other is compared, chiefly in cattle. Laminar bone has a more intimate blood-supply than haversian bone, and it has a larger surface area of blood-channel per unit volume. The volume of channel per unit volume is about equal in the two types of bone. The mean distance between anastomoses is less in laminar than in haversian bone. The cement-line round haversian systems hinders the passage of materials through the canaliculi. The osteocytes in the interstitial lamellae are thus in a worse position for obtaining foodstuffs and disposing of waste products than osteocytes in laminar bone a similar distance from the nearest blood-vessel.

It is suggested that the formation of haversian systems in laminar bone interferes with the latter's blood-supply and leads to the formation of further haversian systems.

INTRODUCTION

ONE of the most characteristic features of human bone under the microscope is the haversian system. Most adult human bone is either in the form of haversian systems or of interstitial lamellae, which are merely the remains of previously existing haversian systems. It is not surprising, therefore, that there is a tendency to regard the haversian system as the unit of bone. Thus Maximov and Bloom say 'The Haversian system or *osteon* is the unit of structure of compact bone' (1957, p. 132). Harris and Ham (1956) say 'The unit of structure of compact bone is the Haversian system'. In fact haversian systems are only the units of structure of compact bone in the sense that a wrinkle is the unit of structure of facial skin. Haversian systems are related to the age of the animal. They are absent from the bones of most vertebrates, are almost absent from some mammals, for instance most of the rodents and the bats, and occur in large numbers in others, for instance the cat, only long after sexual maturity has been reached. It is not very meaningful to talk about

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the unit of structure of bone at all, and if we must, we should seek it at the collagen/bone-crystal level, and no higher.

Many authors have described in mammals types of bone that do not contain haversian systems (Gebhardt, 1901; Mátyás, 1929; Foote, 1916; Enlow and Brown, 1957; Brouwer, 1952). The purpose of this paper is to describe more fully a particular type of bone lacking haversian systems, and to compare some of its characteristics with those of bone full of haversian systems (which from now on I shall call 'haversian bone'). This type of bone has been given several names: Gebhardt (1901) calls it *in toto konzentrische Knochen;* Enlow and Brown (1957) call it 'plexiform bone' but do not describe it very fully, and to some extent have misinterpreted it. Brouwer calls it *vaatnetten* (wire-netting) bone, an apt name. I intend to call it 'laminar bone', because in cross-section it can be seen to consist of a series of laminae.

RESULTS

Laminar bone

The description that follows is of bone lying in the anterior quadrant of the mid-shaft of the femur of mature cattle.

Laminar bone consists of a series of concentrically arranged laminae. Each lamina curves round the femur, having about the same radius of curvature as the whole bone, and therefore tending to go right round it. Many laminae are arranged one outside the other, producing parts of a series of co-axial cylinders. The thickness of each lamina is more or less constant throughout its extent, and the thickness of different laminae is very similar. In cattle it is about 0·18 mm, though in parts it may become as much as 0·21 mm. The measurements referred to here and elsewhere in the paper are of undecalcified sections, so distortion must be very slight. Between each lamina and the next is a net-like system of blood-vessels, which exists essentially in only two dimensions. The blood-vessels branch and anastomose very freely. Fig. 1 is a camera lucida drawing of part of a tangential section of the femur of a pig. Corresponding views of cow's bone have a similar appearance, except that the cow's network has a slightly larger mesh than that of the pig. Now for blood to pass freely through the bone there must be connexions between one blood-network and the next, and these, of course, exist. They do not, however, occur very frequently, although where they do they have a larger bore than the channels forming the network between the laminae. We may compare the whole structure to that of a large hotel. On each floor there is a system of rather narrow corridors, while between the floors there is a relatively small number of lifts and staircases. Where the connexions occur they tend to pass straight through many laminae in a radial direction. The analogy with the hotel still holds, since staircases usually occur in the same relative place in various floors, and do not start at random in the corridor network.

The histological structure of each lamina is not simple. Each is divided

to three zones (fig. 2). Starting from the endosteal side, the first extends
om the plane of the blood-network to about one-third of the way across the
mina. It consists of lamellar bone whose osteocytes are flattened in the
dial axis and lengthened in the tangential and longitudinal axes. Lamellar
ne, the *lamellarer Schalenknochen* of German histologists, is usually re-
acement, that is, secondary bone;
e fibres show a high degree of
ientation and it consists of very
in lamellae (about 5 μ thick). The
llagen fibres in each particular
mella are usually all orientated in
e same direction, the orientation is
ually different from that of the
rils in the neighbouring lamellae,
d the degree of calcification of the
ne is different in neighbouring
mellae (Engström and Engfeldt,
53; Rouiller, 1952). Lamellar bone
to be contrasted with woven bone
aserknochen), in which the collagen
rils are thicker and matted, and
e cells are more globular and
ger, and have fewer canaliculi.
itchard (1956) says that lamellar
ne is 'essentially a *replacing*, not a
imary tissue'. In fact in laminar
ne we have an example of lamellar
ne that is primary. The lamellar
ne extends about one-third of the
stance between one blood-network
d the next. It then gives way
woven bone. There is no cement
e between the two types of bone,
ch as is found round the outside of
versian systems, but the change-
er is sharp. This woven bone extends for about another third of the inter-net-
rk distance. It is, however, interrupted in the middle by a line that in or-
nary light appears to be bright. This is the *blasse Mittellinie* that v. Kölliker
mologized with the cement line round haversian systems (1889). A high-
wer view shows that the brightness is due, in part at least, to the fact that
ere are many fewer canaliculi crossing this particular part of the bone than
ere are elsewhere. Canaliculi appear dark in air-filled sections, so that this
ea without canaliculi lets through more light than the rest of the bone.
cause few canaliculi pass across the bright line, it is apparent that each
od-network will be mainly responsible for the nourishment of the half

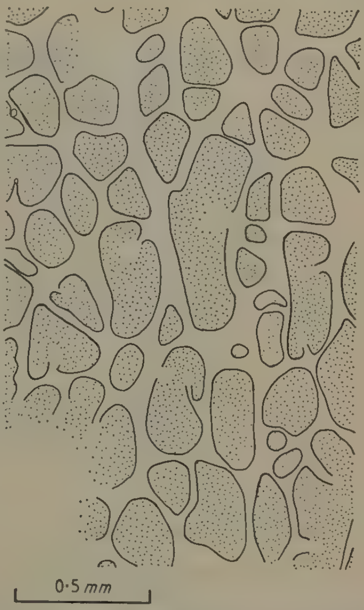

FIG. 1. Camera lucida drawing of a tangential section from the femur of a pig. Bony tissue is stippled. There is a two-dimensional network of blood-channels. In some places accurate drawing is impossible, where channels pass out of the plane of the section, and this is represented by gaps in the stippling.

lamina on each side of itself. Finally, there is another layer of lamellar bon before the next blood-network is reached.

There is a considerable amount of variation between various parts of bones and more particularly between species, in the amount of woven bone in eac lamina. In some cases it extends from one-third of the way across right u

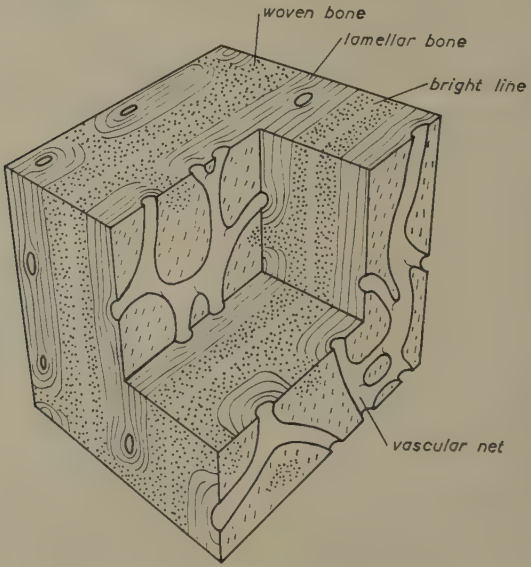

Fig. 2. Three-dimensional diagram of a piece of laminar bone showing parts of the vascular network of two laminae.

to the edge of the blood-network on the periosteal side. In other cases th lamellar layers are so thick that there is only one row of woven-bone osteocytes. In general, however, the woven bone occupies just over one-third o the total thickness of the lamina.

Laminar bone is not formed continuously and smoothly during the growt of the animal, but in a series of spurts. In sections of developing lamina bone it is possible to see various stages in this growth process, though it i not possible to follow the development of any particular lamina. I hav examined the bones of cats, man, and cattle, and in all species the mode o formation seems to be the same, although details, particularly of scale, var Fig. 3 shows, very diagrammatically, the sequence of events.

(*a*) The periosteum, lying outside a layer of woven bone, has blood-vessel on the inner surface of the fibrous layer, lying on the sub-periosteal surfac of the bone.

(*b*) A large amount of woven bone is formed very quickly. The new laye of bone is supported, and held clear of the old sub-periosteal surface, by a occasional bridge of woven bone. The blood-vessels that previously lay on the sub-periosteal surface of the bone are now enclosed in spaces completely surrounded by woven bone. Presumably the fibrous layer of the periosteum with some of its osteoblastic layer withdraws from the surface of the bone, except in the region of the bridges, and then very quickly lays down bone on

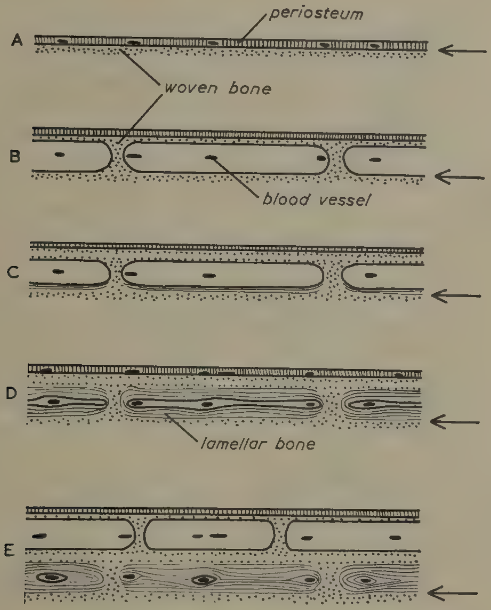

FIG. 3. Diagrammatic representation of the formation of laminar bone. The arrows show the position of the original bone surface.

ts own sub-periosteal surface. I have never seen the periosteum slung between wo bridges without any bone on its inner surface. This is not surprising, s it is likely that this stage lasts a mere matter of hours, and I have often een the periosteum with a very thin layer of bone, only about 4 μ thick, on ts inner surface.

(*c*) Lamellar bone begins to form on the endosteal side of the cavity in vhich the blood-vessels lie, the osteoblasts probably coming from part of he original periosteum, left behind and enclosed in the cavity in the woven one. At or about this time the periosteum has another phase of activity and nother thin layer of woven bone is laid down. This would not be remarkble were it not that the 'bright line' is found at the junction between the wo layers of bone formed by the periosteum during the stages (*b*) and (*c*). As

I have said above, the main characteristic of the bright line is that very few canaliculi pass through it, and from the point of view of the nutrition of the cells, the limits of each lamina are the bright lines. Therefore this phase of activity of the periosteum in stage (*c*) can be considered to be the beginning of the formation of a new lamina.

(*d*) The spaces round the blood-vessels get smaller as more lamellar bone is laid down on the walls of the cavity.

(*e*) The cycle of periosteal activity starts again, before the lumina of the previously-formed cavities have been filled in. Sometimes the periosteum is so active, compared with the speed of the infilling of the cavities it leaves, that there may be as many as four series of cavities extending into the wall of the shaft, becoming less spacious the deeper they are in the bone.

A particularly important feature of the formation of laminar bone would seem to be that it is a way of increasing the thickness of a bone quickly, while still having a large proportion of the bony substance in the form of lamellar bone. A rather substantial framework of woven bone is built quickly, and then the spaces are filled with lamellar bone at a more gentle pace. As lamellar bone is found in the higher vertebrates, it is probable that it is superior to woven bone in some respects, possibly in its strength.

Laminar bone is found principally in artiodactyls and in some of the ornithischian and saurischian dinosaurs. In these it is found mainly in the compacta of the long bones. It is also, however, found in some other groups of mammals at some time in the life-history. Human bone, for example, does not universally grow in width by the formation of laminar bone, but it does in certain parts of the skeleton, and at certain times of life. Fig. 4 shows a camera lucida drawing of a tangential section of subperiosteal bone from the shaft of an adult human femur. It shows the net-like appearance of blood-channels in typical laminar bone. However, in this particular piece of bone the laminar bone was only about one lamina thick. Deeper than this the bone was rapidly converted into haversian bone by erosion and redeposition. In general it can be said that in many mammals larger than the rat some bones grow in thickness, at least at some stage, by the formation of laminar bone. The extent to which this bone is replaced by haversian bone, and also the extent to which this method of bone formation is replaced by some other such as, for instance, the simple accretion of sub-periosteal lamellae in the cat, varies enormously from species to species.

The measurement of vascularization in laminar and haversian bone

Bone is a very hard substance, and, in contrast to other tissues, post-mortem changes do not affect the size and relationships of the channels within it. Therefore it is legitimate to make ground sections, perform measurements on them, and assume that these measurements would have been the same in life. Of course, the channels found in bone are not completely filled with blood; there will be blood-vessel epithelia and nerves and perhaps occasionally lymph vessels as well (Rutishauser, Rouiller, and Veyrat, 1954), and therefore measurements, particularly of volume, should be reviewed with this in mind.

I have taken an ox femur and examined areas of laminar bone and haversian bone from the same level, and have made histograms, showing the distances separating points, taken at random, from the nearest channel. This gives an estimate of the density of the blood-supply in the bone; finally I have calculated the area of channel wall in a cubic centimetre of bone, and the volume of channel in a cubic centimetre.

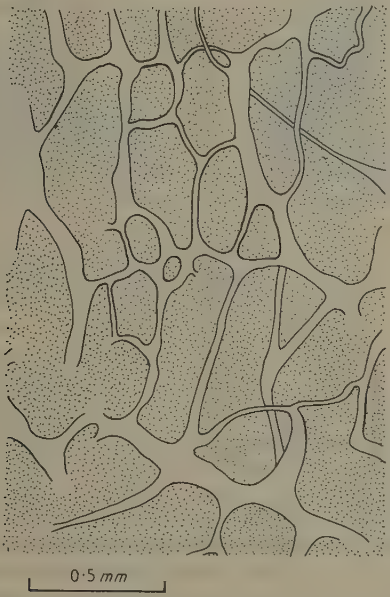

FIG. 4. Camera lucida drawing of a tangential section from the femur of a man, taken very close to the sub-periosteal surface of the bone.

Distance of random points from the nearest blood-vessel. For haversian bone the calculation was simple. A number of transverse sections about 120 μ thick were ground between ground glass plates in alcohol and mounted in solid balsam. A section was put under a vernier microscope and moved around at random. The distance from the intersection of the eyepiece cross-wires to the nearest channel was then measured. The osteocytes were arranged fairly evenly and very densely through the tissue, so that it was not necessary to measure the distance from any particular cell to the nearest channel, a process that would have introduced an element of subjective choice. This method would have been unsuitable if there had been areas of bone with a lesser density of osteocytes. It might be objected that a cross-section of a portion of haversian bone does not give a true picture of the spatial relations between the osteocytes and the channels, in that an osteocyte that was apparently a long way from the nearest channel might be quite close to one that was just out of the plane of the section but running parallel to that plane. This is unlikely to have materially affected the results for two reasons.

(1) Observations were made on cleared decalcified blocks of bone that had been filled with Indian ink by evacuating them under the ink (Ruth, 1947). It could be seen that the great majority of the haversian systems ran more or less parallel to the long axis of the bone, though not exactly so.

(2) The thickness of the sections was roughly the same as the maximum distance between an osteocyte and the nearest channel. Now the measurements were made from an axis that ran at right angles to the plane of the section, to the nearest point that a channel approached this axis. Any measurement really refers to a *row* of osteocytes arranged along the axis of observation. Therefore only when the measured distance was large *and* when there was a channel just outside the plane of the section would the measurement not be representative of the row of osteocytes as a whole, and even then there would be many osteocytes in the row for which the measurement would be appropriate. Therefore this method of measurement would tend slightly to overestimate the distance in some few cases, but only very rarely.

The type of measurement outlined above is not appropriate for laminar bone, because the ground sections do not show the channels between the laminae at all clearly, except where they come to the surface of the section. Instead, measurements were taken from a *tangential* section, and these measurements were then converted arithmetically, by assuming that the cells were arranged evenly between the laminae, an assumption that observation shows to be substantially correct. Thus a point appearing to lie say 0·05 mm from the nearest channel, when viewed in tangential section, could be considered to lie 0·01 mm, 0·02 mm, &c., from the plane of the network of blood-channels, or to lie actually in the plane of the network. This arithmetical conversion was made simpler by the fact that the interlaminar distance is almost constant. Twenty measurements showed one distance of 0·21 mm, one of 0·19 mm, and the rest of 0·18 mm or 0·17 mm. For the purposes of the conversion the distance was taken as 0·20 mm. This reduces the apparent vascularity slightly. The conversion was performed as follows. Every reading of the distance from the nearest channel was assumed to apply to 11 points, respectively 0 mm, 0·01 mm, &c., up to 0·1 mm, which last value is just half-way across the lamina. The hypotenuses of the right-angled triangles whose other sides were (*a*) the distance from the nearest channel as measured in the plane of the network, and (*b*) the assumed distances from the plane of the network, were calculated. Therefore every original measurement was converted into 11 measurements. The final number of points at each particular distance was added up, divided by 11, and put on the histogram. A few extra readings were made to allow for the fact that one in 11 of the points pearing to lie on a blood-vessel would be placed, by calculation, in the ood-vessel. Two hundred measurements of each type of bone were made. he histograms (fig. 5) show the general character of the results. They can

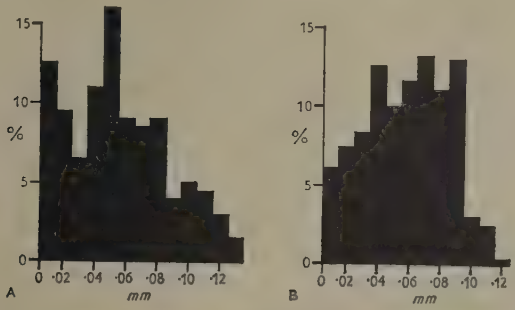

FIG. 5. Histograms showing the distribution of distances separating points taken at random from the nearest blood-vessel in the femur of cattle. Abscissa: distance from the nearest blood-vessel. A, haversian bone; B, laminar bone.

so be expressed in this way:

istance from the nearest blood-channel that	*50%*	*90%*	*100% osteo-cytes lie within*
versian . . .	0·06 mm	0·11 mm	0·14 mm
ninar . . .	0·07 mm	0·10 mm	0·13 mm

Surface area of channels. For haversian bone this calculation is again ite simple. If we assume that the haversian canals run straight up and wn the length of the bone, then we need to know the mean diameter of e canals and their density in the bone. One hundred measurements of the ameter were made. The mean was 0·022 mm. Forty measurements were ade of the number of haversian systems appearing in a field of view when e slide was moved around at random. This value was remarkably constant; e mean number was 8·43, the greatest value 11 and the smallest 5. From is it could be calculated that there were 34·3 haversian systems per square m. The mean circumference is $\pi\times 0{\cdot}022$ mm, and the total area of channel ll per c.c. will be $\pi\times 0{\cdot}22\times 34{\cdot}3\times 100\times 10$ sq. mm. That is 2,370 sq. mm. 23·7 sq. cm per c.c. However, this value is based on the assumption that haversian bone the only channels are in haversian systems running more less straight up and down the bone. There are in fact anastomosing vessels at run from one system to the next. They usually have a somewhat smaller ameter than the main channels. Their total length in a given volume of ne is much less than that of the haversian channels. As I shall show below eir length is about 12% that of the main haversian systems. If we assume that their diameter is the same as that of the main channels, we must increase the value for the area of channel by 12%. That is, the value is no 26·6 sq. cm. I should mention here that wherever simplifying assumption have been made, they have been made so as to increase the apparent efficiency of haversian bone, and to decrease that of laminar bone.

For laminar bone the calculation is again more complicated. The metho used was to obtain the area of channel in each network, and to multiply b the number of laminae occurring in a centimetre thickness of wall.

A tangential section was made of the bone, and a camera lucida drawin was made on to thick typing paper. This was cut out and the paper representing channel and that representing not-channel was weighed. The weigh of the paper representing channel was 25·1% of the whole, which was rounde to 25%. As a first approximation we can assume that the channels have n thickness but are two-sided. Therefore in each square centimetre of blood network there will be 0·5 sq. cm of channel surface. The channels are i fact elliptical in cross-section, with the minor axis in the radial plane. On hundred measurements were made of the thickness of the lumen of th blood-vessel in the radial direction. These were made at the point wher the channel left the plane of the section where they could be accuratel measured. The mean thickness was 0·013 mm. The major axes of the ellipse could not be measured directly as they did not show up clearly in the sectio The width of the blood-vessel was calculated from observations made o tangential sections. Here choices had to be made because, although th channels had a fairly uniform width, when seen in tangential section the tended to widen out where they anastomosed. The narrower portions wer taken as being typical. Forty measurements were taken, and the mean valu was 0·033 mm. The channels can be considered then to be elliptical in section, with a proportion between the major and minor axes of 33:13. No the circumference of an ellipse of this shape is 79 units. We have previousl assumed that the area was double the area visible in tangential view. Th latter area was the sum of the areas of vessels that were, in fact, about 0·33 m across. That is, we have assumed that for a unit length of tube the area i $1\times2\times33 = 66$ units. In fact it should be 1×79. We must increase ou original estimate by 19·5%. This produces a value of 0·5975 sq. cm of blood channel surface in one square cm of blood-network. Inspection showe that there were on the average 55 laminae in a centimetre thickness of wal Therefore the value for one cubic cm of bone is 32·9 sq. cm. This valu ignores the value of the anastomosing vessels running between successiv laminae, but these are comparatively rare.

Volume of channels. The value for haversian bone is $n\times\pi\times r^2\times L$, wher n is the number of haversian systems per square cm and L is the length o one haversian system in one cm length of bone, that is, 10 mm. From th information we have already obtained we can calculate that this value i 13·04 cu. mm. per c.c. Allowance must then be made for the anastomosin vessels, which will increase this value by 12%, making the final value 14·

cu. mm. per c.c. Therefore about 1·46% of haversian bone is occupied by channels.

In laminar bone the cross-sectional area of the channel is $\pi \times a \times b$, where a and b are the major and minor semi-axes respectively. These have the values 0·0165 mm and 0·0065 mm. The mean cross-sectional area is therefore 0·00033693 sq. mm. Now the thickness of each channel in the tangential plane is 0·033 mm. If therefore we consider the channels to run in parallel rows straight up and down the axis of the bone we can calculate the length of channel per square cm of section. There will be one channel for every $\frac{0{\cdot}033 \times 100}{25}$ mm, that is every 0·132 mm. Therefore in a cm square in the plane of the network the total length of channel will be $\frac{10}{0{\cdot}132} = 75{\cdot}75$ cm. Now there are 55 laminae per cubic cm; therefore the total length per c.c. will be $75{\cdot}75 \times 55$ cm, that is 4,166 cm. Since the mean cross-sectional area of the channels is 0·00033693 sq. cm, the total volume of channel will be 0·014 c.c. per c.c. of bone.

In summary, for ox bone:

	haversian	*laminar*
volume of channel per c.c. of bone . .	1·46%	1·40%
area of channel " " " " . .	26·6 sq. cm.	32·9 sq. cm.

The measurements and calculations given above are, of course, rather crude, and cannot give more than a fairly good idea of the conditions in life. Nevertheless, some points of interest emerge. The total volume of channel in the two types of bone is surprisingly similar. The difference is so small (laminar bone has about 4% less volume of channel than haversian bone) that we cannot decide whether it is significant or not. However, the difference in the area of channel is probably real, as the laminar bone has about 24% more area of channel than haversian bone. Finally, the histograms show that although there is not very much difference in the distance separating the further cells from the nearest channels, yet, in this respect also, laminar bone is slightly more efficient than haversian bone. It is interesting to compare the histograms for cattle with those for the pig (fig. 6), the method of observation being the same in both cases. It can be seen that in the pig the laminar bone is more efficient than the haversian bone by a considerable extent, if we consider the bone to be more efficient the fewer cells it has a large distance from the nearest blood-vessel. This is because although the density of haversian systems is about the same as in ox bone, in fact slightly less (28 per sq. mm.), the number of laminae per cm is much greater, as there is a lamina about every 0·1 mm, compared with every 0·18 mm or 0·19 mm in ox bone.

The effect of the interruption of canaliculi. The canaliculi of an osteocyte anastomose freely with those of neighbouring osteocytes, and in this way materials are passed from one cell to the next. However, these canalicular connexions are not continuous throughout the bone because they are interrupted at the cement lines in haversian bone, and at the 'bright line' in laminar bone. This interruption is not complete, since some canaliculi do pass across these boundaries, especially in laminar bone, but the great majority do not. As a result the flow of materials from one side of the border to the other will be very greatly reduced. We must consider what effect this interruption will have in the two kinds of bone.

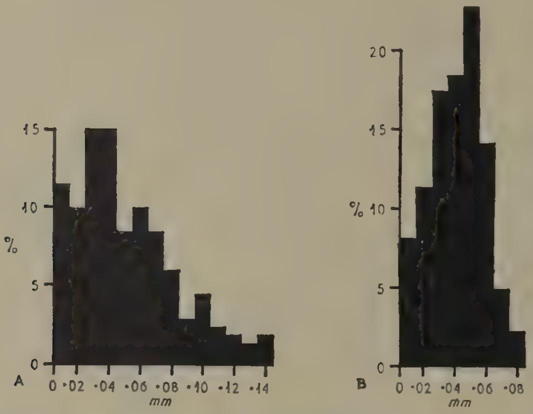

FIG. 6. Histograms showing the distribution of distances separating points taken at random from the nearest blood-vessel in the femur of pig. Abscissa; distance from the nearest blood-vessel. A, haversian bone; B, laminar bone.

In laminar bone the effect will be small. The bright line is placed about midway between the two vascular networks on each side of it. Therefore there is no interruption of the canaliculi between a cell and its nearest blood-vessel. In haversian bone, however, cells in the interstitial lamellae will be parted, not only from the nearest blood-vessel, but from *all* blood-vessels. It is clear then that histograms merely showing the distance of cells from the nearest blood-vessel tend to belittle the differences between the worst placed cells in the two types of bone.

There is another effect, of smaller importance than that mentioned above, that should be taken into consideration. Suppose that a cell or small group of cells adjacent to an interruption, but inside the haversian system in haversian bone, has a large amount of waste product that must be removed, and suppose further that the concentration of waste matter is higher in this group of cells than elsewhere. In laminar bone the particles of the waste product will be able to diffuse in any direction on one side of the effectively *plane* interruption. In haversian bone, however, the paths of diffusion are much more confined, being restricted to the inside of a cylinder. The same

onditions can be envisaged, of course, with a local deficiency of some pro-
uct, only now the material would be flowing towards the point. This effect,
owever, will probably be of small importance, because it will only happen
hen there is a localized deficiency or surplus of some material, and this
ate of affairs is probably not very common in bone.

Frequency of anastomoses. Presumably the health of the cells in bone de-
ends on the continual flow of blood through the capillaries in the bone.
the blood-supply to the whole bone is interrupted, then the whole bone
ill suffer, but if the flow is interrupted at any one particular spot, then
nly the cells in the surrounding bone will suffer. How much of the bone
ill suffer will depend, among other things, on the nature of the capillaries
the bony channels on each side of the interruptions, and the frequency of
astomosis between the channels.

If there is only a single capillary in the interrupted channel, then the
ood will be in a state of stasis as far back as the first anastomosis with another
ny channel in which the blood is still flowing. In general, however, it
ems that there are usually at least two blood-vessels in the channel (Ruti-
user, Rouiller, and Veyrat, 1954). If there are two capillaries carrying
ood in opposite directions, then after a while the ends of the capillaries
ay be able to join and re-establish flow. This would probably take some
ne, however. Therefore, if an obstruction occurs in a bone-channel, blood
ll for some time be stagnant as far back as the next anastomosis until fresh
pillary connexions have been made. The shorter the distance between
y two anastomoses in a bone vascular system, the less will be the likeli-
od of any cells dying as a result of the interruption of flow at any one
int, and even if cells do die, the volume of bone in which the cells die will
smaller if the anastomoses are more frequent.

I have made frequency histograms of the inter-anastomosis length in
ninar and haversian bone of cattle, in order to test the relative efficiency
the two types. As with other measurements, haversian and laminar bone
ve to be treated differently. The two kinds of bone came from the femur
the same level as before. The haversian bone was treated as follows. It
s degreased, dried, and filled with Indian ink by evacuating the air from
while it was immersed in the ink. It was decalcified and then cleared in
cerine (Ruth, 1947). The decalcification caused a reduction of all dimen-
ns of about 5 to 7%. This has been taken into account in constructing
histograms. The block of bone was then cut into slices about 1 mm
ck, and drawings of the blood-channels were made by means of a camera
ida. Most of the channels in the bone show up extremely clearly, but not
of the finer anastomosing channels are filled with the ink. Where they
ve the main channel each smaller tube can be seen by the small black
nple that its base makes on the main channel wall. Careful observation
this point always showed the course of the anastomosing tube. The ma-
ity of the haversian canals ran fairly straight, parallel to the main axis
the bone. At frequent intervals cross-tubes, usually rather small, run

from one canal to the next, commonly about at right angles to the long axis of the bone. When the pattern of the channels had been drawn, measurements were made of the distances between the anastomoses. All channels were measured along their length; curving channels were measured as part of the circumference of a circle, and not as the chord to this. On the histograms I have attempted to distinguish between the main tubes and the anastomosing ones by putting them into two classes according to whether they made an angle of more or less than 45° with the long axis of the bone. The main tubes and the cross-connecting ones do not fall into two distinct classes. It is not possible, therefore, to distinguish between them qualitatively, and a quantitative measure must be used. The apparent angle of a channel would of course appear to be less, the more the main axis of the bone and the divergent tube come to lie in a plane parallel to the line of sight, but manipulation of two rulers held at an angle to each other will show that the apparent angle is never *greater* than the actual angle, though it may well be less, so long as one of the rulers, representing the long axis of the bone, is at right angle to the line of sight. This condition is fulfilled in the case of the observations mentioned above, and so all angles classed as being more than 45° will be so in fact, though some of those classed as less than 45° will in fact be more.

To obtain a histogram for laminar bone a thin section was ground paralle to a tangent to the shaft, so that a vascular network lay in the plane of the section. The network was drawn by means of the camera lucida, and measured as before. In laminar bone it is not very useful to distinguish between those channels lying parallel to the long axis of the bone and those lying at an angle to it; although there is some tendency for the channels lying parallel to the long axis of the bone to be longer than the others, there is in no way the distinction found in haversian bone. In the histograms for laminar bone the anastomoses *between* networks have been ignored. They have a length about equal to the laminar width, which, in cattle, is about 0·18 mm. These channels are, as I have mentioned before, comparatively sparse and have a greater bore than the channels lying in the plane of the network.

The histograms (fig. 7), each representing 200 measurements, show tha there are clear-cut differences between the distributions of inter-anastomosis length (that is the distance between the anastomoses) in the two types of bone. In laminar bone the inter-anatomosis length was never as great as 0·4 mm, while in haversian bone a length more than 0·9 mm is found. Moreover, the modal length in haversian bone lies between 0·1 and 0·2 mm while in laminar bone it is less than 0·1 mm. Finally, it can be seen that the anastomoses between the haversian canals make up a fairly large proportion of the distances less than 0·2 mm, about 40%.

Histograms of other bones (fig. 8) show that cattle are not peculiar in these differences; histograms of 200 readings from the femora of man and the cat and from the rib of a rabbit show patterns characteristic of haversian bone, with a long tail towards the higher values, while laminar bone of the

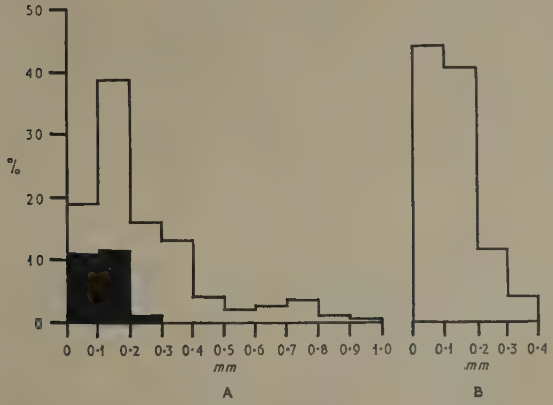

FIG. 7. Histogram showing inter-anastomosis length of channels in the femur of cattle. A, haversian bone; B, laminar bone. In A the black part of the histogram represents cross-connecting channels. Abscissa: length of inter-anastomosis distance.

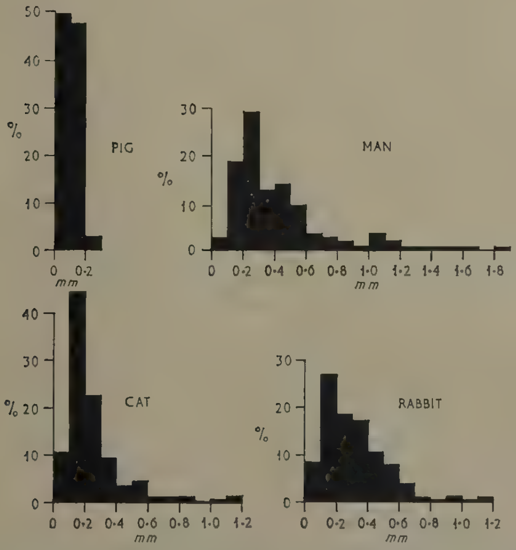

FIG. 8. Histograms showing anastomosis length of channels in various species. A, laminar bone from femur of pig; B, haversian bone from femur of man; C, haversian bone from the tibia of a cat; D, primary bone from the rib of a rabbit.

pig has a histogram like that of cattle. It should be noted, however, that the rabbit has very few haversian systems. Instead it has channels whose spatial arrangement can be seen by examining decalcified blocks to be similar to that in haversian bone, but these channels are in fact primary, that is to say they have been there since the bone was first formed, whereas, as I have mentioned above, haversian canals are formed as the result of the filling in of a larger cylinder eroded in the bone.

DISCUSSION

When we see bone of different histological type in different mammals, and in different parts of the same bone, we naturally wonder why these differences should exist. I have compared different properties of the two types of bone in an effort to see if they can be related to the different needs or uses of the bone in the animals. In a previous paper (Currey, 1959) I have shown that haversian bone in cattle is weaker in ultimate tensile strength, when tested along the long axis of the bone, than primary bone from the same femur and from the same level in the bone. In this paper I have considered possible differences of vascularization between laminar bone and haversian bone. It should be noted that the comparison is made between different pairs of histological types in the two cases. In testing ultimate strength I compared primary with secondary bone. The secondary bone has all haversian bone, but although most of the primary bone was laminar there was a certain amount containing primary osteones, and this seemed as strong as typical laminar bone. In the present paper I am comparing well-developed, mature haversian bone with typical laminar bone.

Harris and Ham (1956) say: '. . . for bone to contain such a good blood supply requires that it be constructed most ingeniously.' They make it clear that the ingeniously constructed bone they are referring to is haversian bone. The 'ingenuity' lies in the fact that 'even in dense bone no cell is generally more than a small fraction of a millimetre from a capillary'. Now if we are considering the intimacy of the connexion between the blood and the cells the blood is supplying, it is reasonable to assume that one of the criteria of a good blood-supply is the distance that the worst-placed cells are from the nearest blood-vessel. Reference to fig. 5 shows that in cattle there is very little difference between the two types of bone in this respect, though there is more difference in the pig (fig. 6, p. 362). However, as I have shown above, the bone cells in the interstitial lamellae are very badly placed as regards nutrition, because they are divided from all blood-vessels by cement lines, which interrupt the continuity of the canaliculi. And it is precisely the cells in the interstitial lamellae that are a long way from all blood-vessels. On the other hand, the badly-placed cells in laminar bone are never separated from all blood-vessels, and, what is more, they are usually not divided from the blood-vessels nearest to them. (This is not strictly true. In some types of laminar bone the bright line is slightly nearer one vascular network than the other.) If, then, we consider the situation of the worst-placed cells, it is clear that laminar bone is more efficient than haversian bone.

It can be argued that the situation of the worst-placed cells is not the best criterion, and that it would be better to consider the total volume of blood in a unit volume of bone. If haversian bone could be shown to have a much larger volume of channel for the blood to run in, then it could be said that the increased steepness of the diffusion gradient would outweigh the fact that the more distant cells were farther away in haversian bone. In fact I have not been able to detect any significant difference. I have not measured the volume of blood-vessel in the bone, but only the volume of the channels in which it runs. The volume of the blood will presumably nearly equal this in times of metabolic stress. Moreover, the surface area of channel per unit volume is actually less in haversian bone than in laminar bone.

Using the criteria discussed above, we can say that laminar bone is either as efficient as haversian bone, or, much more probably, is more efficient. I have made some assumptions when using the word 'efficient' that should be made explicit. I have assumed that the volume of blood-vessel in the bone is as little as is necessary to perform its function. That is to say I have assumed that the amount of blood flowing through the bone is not so great in all metabolic states as to make differences in volume of channel, &c., of merely academic interest, and that in some circumstances the efficiency of the vascularization of the bone may decide whether a portion of the bone will die or not. The second assumption that has been made is that it is advantageous for bone that the cells within it should remain alive and healthy. This might appear obvious, but in two groups of vertebrates, the heterostracan Agnatha and the teleosts, there is bone without bone cells. Nevertheless, the bone of the majority of vertebrates does contain bone cells, though what their exact function is remains obscure. To some extent it appears to be related to the maintenance of bone. Pritchard (1956) writes: '. . . the osteocyte is an essential constituent of bone, for when the cells die the matrix around them, although persisting for a time, eventually crumbles and is removed to be replaced by living bone. Osteocyte death is, in fact, synonymous with bone death.' This second assumption would seem, then, to be justified.

In short, it can be said that two out of the three ways of comparing the vascularization of laminar and haversian bone show that laminar bone is more efficient, and that the third, based on the volume of channel, shows effectively no difference. The question of frequency of anastomosis is somewhat different. If the blood flow through the bone is unchecked, then the frequency of anastomosis is not very important. If occlusion of one of the channels occurs, however, a short inter-anastomosis length will be a considerable advantage, as the amount of bone affected by the stasis of the blood will be smaller. Here again laminar bone appears to be considerably more efficient than haversian bone. Ham and Harris (1956) claim that the death of bone cells near the site of a fracture in bone is due to the interruption of the blood-vessels at the fracture site and to the subsequent stasis, and they think that this will affect cells as far back as the next anastomosis. They add: 'In the rabbit's rib, dead bone, as indicated by the lacunae which formerly contained osteocytes becoming empty . . . can be observed for distances ranging up to a millimetre or more from the site of the fracture.' McLean and Urist (1955) say: 'In a large bone of adult man, with a closed non-comminuted fracture, at least 0·5 cm of the shaft is damaged above and below the fracture line.' Although they do not state whether 'damage' involves cell death, this is presumably so. It is interesting, in the light of these remarks, to examine histograms of inter-anastomosis length for the rib of a rabbit and for the femur of a man (fig. 8). In the rabbit rib 90% of the inter-anastomosis lengths were less than 0·6 mm. In man 90% of the inter-anastomosis lengths were less than 0·9 mm. Therefore, if the bone broke at the worst possible place, that is actually at the point of anastomosis, only 10% of the bone should be affected by stasis farther back than 0·6 mm in the rabbit and 0·9 mm in man. On the average the break is likely to occur half-way between anastomosis, and therefore it would be more accurate to say that less than 5% of the bone would be affected by stasis as far back as the figures mentioned above. In haversian bone, and in the primary bone of the rib of the rabbit, the majority of the longer channels run straight up and down the long axis of the bone, so the *volume* of bone that will be affected by stasis will vary according to the angle between the plane of the fracture and the long axis of the bone.

It seems, then, that the mere length between anastomoses is not great enough, either in the rabbit or in man, to be a sufficient explanation of the dying back of the bone for the distances observed by McLean and Urist, and by Harris and Ham. This is not surprising. When a bone is stressed, work is done on it, and it gains a considerable amount of energy. When the bone breaks this energy is suddenly released, and it is probable that this sudden release of energy, as shock waves, &c., will harm cells in the surrounding tissues, including those in the bone itself. The fracture, then, may have effects beyond merely causing the circulation to cease in the neighbouring blood-vessels. Nevertheless, inter-anastomosis length does give an estimate of the least possible volume of bone that will be killed in the event of a fracture, even if, especially in the case of man, it is a considerable underestimate.

This property of haversian bone, that it has a less efficient vascularization than laminar bone, has an interesting consequence. Laminar bone will tend to be stable but haversian bone will not. Examination of cross-sections of haversian bone shows that the systems are not arranged at random. In the bone of cattle haversian systems, when they appear in the previously primary laminar bone, very often occur in rows, each system having one or two close neighbours. These rows are usually arranged along the length of the vascular networks, that is to say they are circumferential; also most haversian systems lie entirely within one lamina, often reaching the bright line on each side, but not crossing them. Now one can explain this either by saying at the bone in that row was of such a kind that it was likely to turn into aversian bone, or else one can say that a localized part of the bone died, as turned into a haversian system, and that this haversian system then fected the bone around it, so that this in turn tended to become haversian

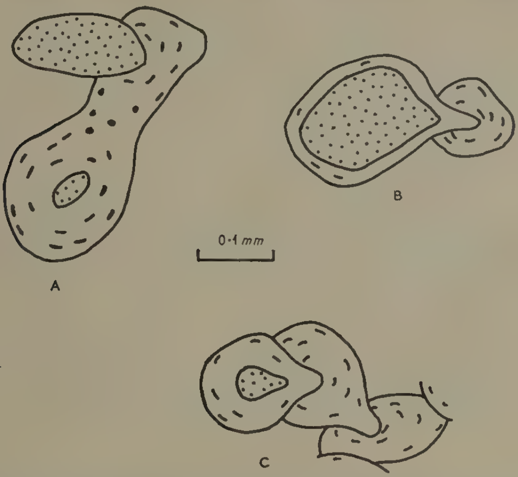

FIG. 9. Three stages in the formation of a new haversian system. A, an erosion cavity, starting from the lumen of another haversian system, is formed. B, bone is laid down on the wall of the cavity. This new-formed bone has now occluded the lumen of the original haversian system. C, many more lamellae have been laid down, and the new haversian system has nearly reached its final form. Three generations of systems can be seen. All these drawings were made by camera lucida from sections taken from the femora of cattle. The resorption cavity is indicated by stippling.

one. While we do not know enough about the causes of the formation of versian systems to exclude the first of these two explanations, it is posble to explain this phenomenon by use of the second. The effect of emedding one haversian system in otherwise laminar bone will be to interrupt e networks and to prevent the free flow of blood through one particular etwork. Therefore the bone just outside the periphery of the system will relatively badly placed and so, if any bone in the area is likely to die, it ill be this part. The haversian systems will tend, then, to replace the priary bone supplied by any one network.

In older haversian bone it is extremely characteristic that one haversian stem will, if it occupies more than a very small portion of the space preously occupied by another adjacent system, send out a 'promontory' to clude the lumen of this adjacent system (fig. 9, c). Now I have shown ove that interstitial lamellae are likely to be particularly badly placed in spect of vascularization. Suppose, then, that some interstitial lamellae adjacent to an haversian system die. This dead bone must be replaced. Now this replacement is only done around a blood-vessel, and so a blood-vessel must reach this non-vascular area. But the only blood-vessels in the neighbourhood are enclosed within the lumina of haversian systems. Therefore erosion must take place on one side of the system to allow the blood-vessel to reach the dead part. When the cavity formed by this erosion is filled in, the original haversian system will be separated by a cement line from its former blood-vessel, which now lies in the centre of an adjacent system (fig. 9). The old haversian system has now become the new interstitial lamellae. Therefore, once haversian systems have formed in a particular area of bone they will tend, by interfering with the primitive blood-supply, to convert more and more of the area into haversian bone.

The questions, on one hand: ‘Which is the more efficient type of bone, haversian or laminar?’ and on the other: ‘Why do haversian systems form in the first place, and why are they more abundant in some animals than in others?’ are entirely different, and I have attempted an answer for part of the first only.

I should like to thank Dr. A. J. Cain, Mr. J. B. Gurdon, Mr. H. K. Pusey, and Mr. T. L. Green for their help and criticism. Part of this work was carried out while I held a Medical Research Council grant.

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A Note on the Innervation of the Integument in Amphioxus, and its Bearing on the Mechanism of Cutaneous Sensibility

By QUENTIN BONE

(*From The Marine Biological Laboratory, Plymouth*)

With one plate (fig. 2)

SUMMARY

Previous conclusions as to the innervation of the integument of amphioxus are iscussed, and it is shown that in addition to the system of free nerve-terminals deribed by Lele, Palmer, and Weddell (1958), the skin of certain regions of the body contains specialized sensory cells and encapsulated nerve-endings. The cells of origin f the free nerve-terminals in the skin are described, and it is shown that they fall into several morphologically different categories. In the light of these observations is suggested that the innervation of the skin of amphioxus does not provide unquivocal evidence for the view that the different cutaneous sensations are determined y different discharge patterns in the *same* fibres of the nerve-rami leading from the kin.

INTRODUCTION

MANY students of the nervous system of amphioxus have investigated the innervation of the skin of the animal; their studies have brought orth a remarkable variety of opinion and suggestion. A good summary of the arlier work has been given by Kutchin (1913). However, general agreement as been reached upon certain aspects of the problem, and it seems clear that the major part of the innervation of the skin consists of free nerve-endings related o the epithelial cells. These endings arise from a sub-epithelial plexus, which n turn is derived from small bundles of fibres given off by the dorsal root ami supplying the skin, which pass through apertures in the thick lamellar asement membranes underlying the epithelium. These apertures are probbly simply irregularities in the lattice-work of fibres making up the basement membrane, and not special tubes, as Langerhans (1876) supposed. The exact manner in which the fine nerve-terminals derived from the subpithelial plexus end in relation to the epithelial cells is disputed. Dogiel 1903) believed that they passed up between the epithelial cells; Retzius (1898) hat they ended in relation to the bases of the epithelial cells. In any case, hey terminate as free nerve-endings.

Thus far there is general agreement, and the most recent contribution to he subject, that of Lele, Palmer, and Weddell (1958), is largely a confirmtion of the earlier observations of Dogiel. These authors added, however, he important observations that the ratio of parent fibres to epithelial cells which they innervated, was as high as 1 : 7, and that the fibres approached the pithelial cells from different directions within the sub-epithelial plexus;

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thus, the innervation of the epithelial cells was patterned at random, each cell receiving terminals derived from at least 2 parent fibres.

There can be little doubt that the majority of the fibres terminating in the skin do so in the way described above. A good description of the arrangement with a useful diagram, has been given by Lele, Palmer, and Weddell. Severa workers, however, have suggested that the situation is complicated by the existence of special sensory cells and special sensory endings.

It is the purpose of the present account to show that within the genera framework of the innervation of the amphioxus integument briefly described above, there are complications, due, first, to the presence of special sensory cells amongst the epithelial cells; secondly, to the presence of complex encapsulated nerve-endings; and thirdly, to the presence of morphologically diverse types of cell within the central nervous system, all apparently giving rise to morphologically (though probably not functionally) similar nerve-endings in the skin.

MATERIAL AND METHODS

The observations recorded in the present account have been made upon whole mounts of adult animals impregnated by the silver methods of Bodian (1936) and Holmes (1947); upon whole mounts of animals stained supravitally with methylene blue, and fixed in ammonium molybdate; and upon polyester wax (Steedman, 1957) sections impregnated by Palmgren's silver-on-the-slide technique (Palmgren, 1948). Animals intended for silver impregnation were fixed either in Bouin's fluid made up with sea-water, or in David's formaldehyde / acetic acid / methanol fluid (David, 1955).

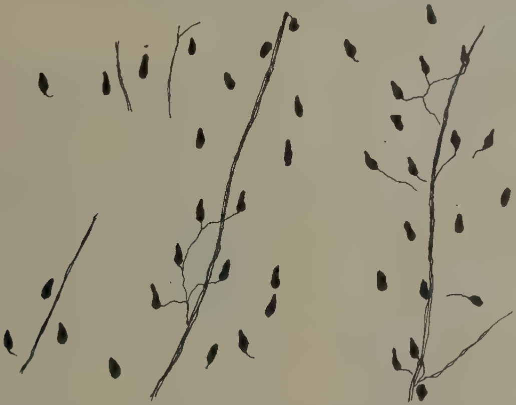
OBSERVATIONS

The sensory cells of the epidermis

Reichert (1870), Langerhans, Dogiel, Joseph (1908), Franz (1923), and Wollenhaupt (1934) all described peripheral sensory cells amongst the normal epithelial cells. Some of these workers believed them to bear small hair-like processes (Reichert called them *Stachelzellen*); all were agreed that they were unlike the normal epithelial cells, and were at least sometimes to be found in connexion with nerve-fibres. Other workers denied the presence of such cells. To the question whether there were special tactile cells, Heymans and van der Stricht (1898) replied 'Nous répondons catégoriquement que non . . .'. Retzius and Kutchin, and more recently Lele, Palmer, and Weddell, also failed to find any cells which could be interpreted as peripheral sensory cells. Lele, Palmer, and Weddell suggest that those authors who believed that they had observed sensory cells amongst the epithelial cells, had been deceived by the differential staining properties of normal and damaged epithelial cells; the so-called sensory cells being merely flattened and compressed epithelial cells. Kutchin supposed that the hair-like processes observed by Reichert were actually some sort of glandular secretion.

It is difficult to rebut the suggestion that damaged epithelial cells have been nterpreted as sensory cells because, as Lele, Palmer, and Weddell point out, very epithelial cell is in connexion with a nerve-terminal, so that even if ne 'sensory' cell appears to be in connexion with a nerve-fibre, this may esult from damage to a normal epithelial cell and its associated nerve-erminals.

However, the weight of the evidence is that sensory cells are found in the ntegument of certain regions of the body, if not over its whole surface. erhaps the most convincing figures of such cells are those given by Dogiel nd by Franz. These authors observed sensory cells in the skin of the head nd tail regions in their methylene blue and haematoxylin preparations.



IG. 1. Sensory cells of rostrum. Whole mount (methylene blue). In some cases, nerve-ores can be traced from the base of the sensory cell to their junction with the nerve rami. he edge of the rostrum and the corpuscles of de Quatrefages are above and to the right. A similar density of sensory cells is found in the tail region.

n common with other authors using silver techniques, I have never observed ny cell which could be interpreted as a sensory cell in the epithelium of lver-impregnated sections and whole mounts. These techniques fail as a ule to demonstrate the abundant sensory cells upon the pterygial muscle Bone, 1960*b*), and it seems therefore that they are inappropriate for the emonstration of such cells. In methylene blue preparations, however, it an easy matter to demonstrate what are almost certainly sensory cells in ertain areas of the skin. My own preparations with this method show many ensory cells in the epithelium of the oral region; in fact, they fully confirm ne observations of Dogiel. I have several times observed in my preparations ppearances exactly resembling his figs. 29 and 30. The sensory cells are rranged in a more or less regular manner over the whole of the skin of the ral hood and snout, this regularity almost certainly excluding the hypo-nesis that these are normal epithelial cells damaged in some manner. The rrangement is seen in fig. 1. Dogiel found these cells in the head region (as did Reichert and Langerhans), and believed that they were to be found also elsewhere in the skin, but less commonly. Franz found them in the tail region, where in my own preparations they are as abundant as upon the rostrum. I have not observed the processes of these cells (which were described most recently by Franz). They are likely to be less evident in methylene blue preparations than in the haematoxylin preparations that Franz (and the earlier workers) used.

It seems most reasonable to conclude that cells of this type are found rarely over the greater part of the body-surface, but that they are more or less abundant upon the head and tail regions. In all probability they are tactile receptors. In addition to these sensory cells, the snout region also contains the well-known corpuscles of de Quatrefages. Their discoverer supposed these corpuscles to be mucus-secreting organs (de Quatrefages, 1845), but it is now generally accepted that they are special sense organs (although there is no evidence for this suggestion). The corpuscles of de Quatrefages appear to consist of peripheral neurones enclosed within a connective-tissue capsule (see Franz, 1923); from conditions in the larval system, it has been suggested that they may form part of a special reflex path concerned with locomotion (Bone, 1960*a*).

In the head region, then, if not elsewhere in the animal, there are two types of sensory receptor, which presumably perform special functions. One of these, the sensory cells described best by Dogiel, probably occurs less abundantly in other regions of the body. The significance of the concentration of sensory elements in the head region will be discussed in a later section of this account.

The encapsulated endings of the metapleural folds

In contrast to the disputes which arose in the literature over the existence of sensory cells in the integument, there has been little consideration of the curious endings which Fusari (1889) reported in the ventral regions of the body. He observed that the cutaneous nerves of the ventral region formed anastomoses, within which he saw nuclei which he supposed to belong to neurones; he spoke of *Ganglienknoten*. Heymans and van der Stricht failed to observe these anastomoses and their associated nuclei. Kutchin observed anastomoses between the branches of adjacent dorsal root rami upon the lateral surface of most of the body (except the head and extreme tail regions). It seems from her description that these anastomoses were not equivalent to those seen by Fedele in the ventral region of the body.

In fact, Fusari observed what are almost certainly complex encapsulated nerve-endings, quite different from anything that had been observed by any other worker. Fig. 2 illustrates these endings from silver preparations. They

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Fig. 2 (plate). Encapsulated nerve-endings from metapleural folds. Both from whole mounts. A, impregnated by Holmes's technique; B, by Bodian's technique. A illustrates a small termination partially in 'side-view' to show relation of the nerve-terminals to the epithelium. B illustrates the complexity of some of the endings of this type.

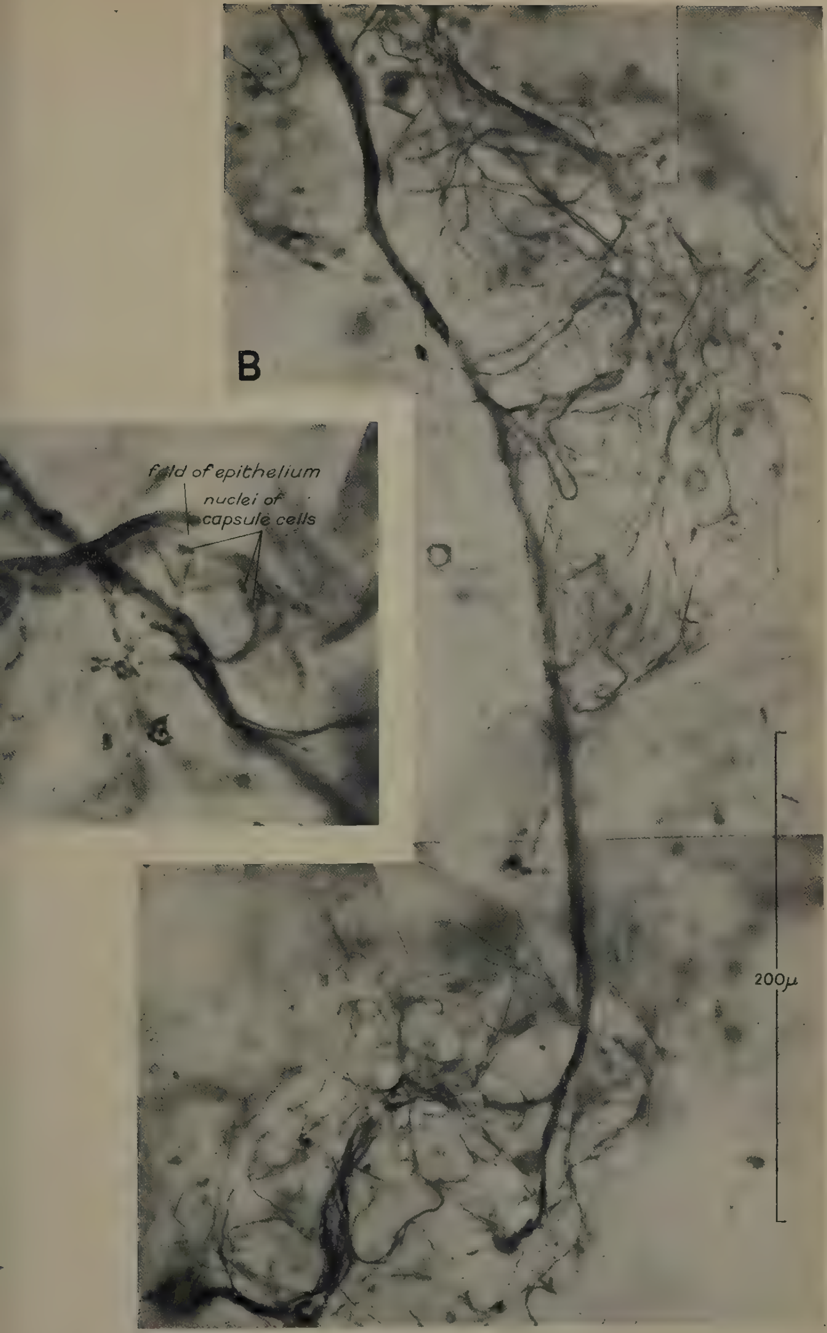

FIG. 2

Q. BONE

are found only (so far as I can observe) in the region of the metapleural folds, and lie just under their outer surface.

The metapleural folds hang down upon each side of the atrial region, and are in life more or less rigid structures, being formed of connective tissue penetrated by a system of cellular canals (analogous to the canals of the rostrum). They appear to act as 'buffers' protecting the pterygial muscle of the atrial floor. The descending rami of the dorsal roots pass into the metapleural folds, where the majority of their constituent fibres end in these encapsulated endings; the remainder recurve and rise up on the inner side of the folds to innervate the skin of the atrial floor under the pterygial muscle. The latter fibres terminate in a sub-epithelial plexus as upon the rest of the body surface. The fibres which terminate in the metapleural folds do not form a sub-epithelial plexus, but end in the endings seen in fig. 2. Often, a single such ending is derived from the fibres belonging to different adjacent dorsal root rami; sometimes single fibres from one ramus may be traced for considerable distances before they terminate in such an ending, which is mainly composed of the fibre-terminations of a different dorsal root ramus. Around each of these endings, and indeed amongst each ending, there are large numbers of nuclei. These are arrayed around the larger loops which are formed by the terminals of a number of fibres, as well as around the smaller fibre bundles and the meshwork where the finest terminals are found. It is improbable that these nuclei belong to nerve-cells, as Fusari supposed. Section of the dorsal root rami leading to these endings results in complete degeneration of the fibres in connexion with them. If the nuclei belong to neurones, then these neurones must be of the vertebrate (or secondary) type, unlike the sensory neurones of the rest of the peripheral nervous system in amphioxus (Bone, 1960*b*). In my opinion, it is most probable that the nuclei around these endings are those of connective tissue-cells which form a capsule around the nerve-terminals.

It is not an easy matter to observe the actual *terminations* of fibres within these endings; for one thing the complexity of the meshwork makes it very difficult to be certain of tracing a single fibre amongst its intricacies, but it seems that the finer terminals lie within spaces formed by the capsules of the connective tissue-cells. Fig. 2, A, illustrates a part of an ending where the skin has been folded, and where the ending is seen in 'side view'; the nuclei lie around the ends of the nerve-filaments where they approach the surface under the epithelium. The resemblance of these endings to the encapsulated endings found in the skin of higher forms makes it very probable that they are encapsulated endings, of a type which has not hitherto been described from amphioxus. Nothing is known of their function, nor, indeed, have they ever been considered in discussions of the responses of the animal to various stimuli. Their histological structure perhaps implies that they respond to mechanical deformations of the integument under which they lie; presumably, therefore, they are activated when the animal is in its normal quiescent position beneath the surface of the substrate. It would be very interesting to have a physiological study upon the region of the body containing these end-organs, comparable to that supplied by Lele, Palmer, and Weddell for the dorsal region of the integument.

The cells of origin of the fibre terminals in the integument

The sensory supply to the skin is afforded entirely by rami of the dorsal root nerves. These roots also contain afferent fibres from peripheral sensory neurones within the atrial system, and efferent viscero-motor fibres leading to that system (Bone, 1960*c*). Fortunately, we are able to identify the viscero-motor cells giving rise to these dorsal root-efferents, and are therefore in a position to state that all the other types of central neurone which send fibres out of the dorsal roots must contribute somatic-sensory afferents from the skin. The visceral-sensory afferents are centripetal fibres from cells within the atrial nervous system. We may presume that the sensory cells within the skin epithelium (at least in the head and tail regions) are primary sensory cells, and contribute centripetal fibres to the dorsal roots.

Now, several quite different cell-types contribute fibres to the dorsal root nerves which terminate in the skin. The chief of these is the bipolar cell figured by Rohde (1888), but first recognized definitely to contribute to the dorsal roots by Retzius (1891).

These cells form two dorsal columns running the whole length of the cord, and send processes to the dorsal roots where they divide into T- or Y-form, one branch passing out of the cord. The abundance of these cells, and their occurrence throughout the length of the cord in both larva and adult, suggest that it is these cells which contribute the majority of fibres to the sub-epithelial plexus, and those terminals end freely in relation to the epithelial cells. In addition to this type of cell, there are at least two others which contribute somatic sensory fibres to the root. The first of these lie in the 'fan' around the exit of the root, with the bipolar cell-body across the central canal and the dendrites relating to the opposite side of the cord to that whence their fibres issue. These cells are abundant (though probably slightly less numerous than the first type of cell described above), and as they occur throughout the length of the cord, probably also contribute fibres to the sub-epithelial plexus. They were figured by Retzius, and by Johnston (1905), who noted that, as they were commissural elements, their relations in the cord contrast with those of the first type. At each root there is a single neurone of a type quite different from the other two described above, whose fibre branches several times before passing out of the root, and whose internal processes relate to the same side of the cord as that on which the cell lies. Fig. 3 summarizes the arrangement of the dorsal root somatic-sensory components; it is quite possible that they may prove to be even more complex, but only the three types of cell mentioned above have been recognized at the present time. As I have considered the arrangements within the central nervous system elsewhere I shall not consider the organization of the dorsal roots in more detail here. It suffices for the present purpose that there are two common types of cell contributing somatic-sensory afferents to each root, and a third type, segmental, that probably is also to be regarded as somatic-sensory.

These types are found throughout the length of the cord. I have been unable to discover any especial type of dorsal root cell in the atrial region, which could perhaps provide the fibres which terminate in the metapleural end-organs. It is evident that whatever may be the peripheral distribution of the fibres of these cells (and perhaps of those of others which may contribute to the somatic-sensory afferents), the sub-epithelial plexus almost certainly contains fibres which are derived from several different types of cell within the central nervous system.

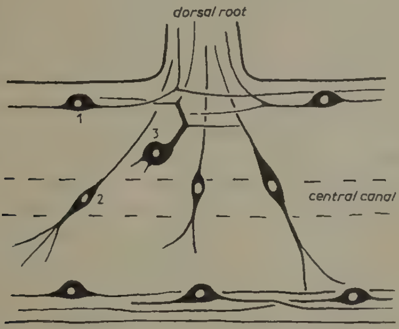

FIG. 3. The somatic-sensory cells of the dorsal root. 1, bipolar cells of Retzius; 2, commissural cells of Retzius; 3, segmental cell. The first two types of cell are much more numerous than illustrated; a few only are shown to avoid complicating the diagram.

If the terminals arising from the sub-epithelial plexus are morphoogically identical, then we are entitled to inquire whether they may be funcionally identical; on the whole, this appears rather improbable. While the ame stimulus may affect the terminals of (let us say) cells of the first and second types, it is certain that the result of the activation of these two ypes of cell will be very different centrally, for the one has its connexions with the same side of the cord as the stimulus, the other with the opposite ide of the cord.

These considerations raise some interesting questions, which will be discussed in the next section.

DISCUSSION

I have attempted to show in the preceding sections that while the greater part of the innervation of the integument of amphioxus probably consists of free nerve-terminals related to the epithelial cells, at least in certain reions of the body special sensory cells and special encapsulated nerve-endings complicate this simple arrangement.

Moreover, so far as the free nerve-terminals are concerned, it seems that these are derived from several different types of neurone which have rather different central connexions.

That the specialized receptors occur in special regions of the body certainly suggests that they respond to some particular stimulus. It is not surprising to find these receptors in the head region, for it is only this region of the body that is normally exposed when the animal is feeding with the greater part of its body concealed beneath the surface of the sand or shell gravel of the sea bottom. The existence of special receptors in the tail is understandable also, for when the animal is disturbed and leaves the substrate, it frequently enters tail first, possibly burrowing as a result of the stimulation of these cells.

The peculiar end-organs in the metapleural folds are more puzzling, particularly as they are presumably activated when the animal is lying at rest in the substrate; although it is not impossible that they afford some information as to the nature of the substrate, there is no evidence for this suggestion.

It seems most reasonable to accept that the specialized receptors in the skin of amphioxus respond to particular stimuli. Their responses will be superposed upon the general background of information which the animal receives from its unspecialized nerve-terminals (when the animal is buried, the free nerve-terminals of the skin must provide a background of information); yet there is no reason to question the view that these free nerve-terminals may respond to the same stimuli as those which activate the special receptors. Indeed, it would appear more probable than not that the mechanism of sensation in amphioxus is not adequately represented by the responses of the special receptors, in a vacuum as it were, with respect to the other types of nerve-ending which may be present in the system. Rather, it seems that the response to a stimulus of a certain sort will be the activation of the receptors especially modified for this type of sensation, together with a general pattern of activity from the other less-specialized nerve-terminals.

The response of the animal to the stimulus will be the result of the central integration of a pattern of discharges from the special receptors and from the free nerve-terminals; not the result of a response to the activity of the special receptors alone.

Thus far our conclusions accord with those of Lele, Palmer, and Weddell. These authors concluded from their histological and experimental studies that the innervation of the skin in amphioxus is not dissimilar to that of the skin and cornea of higher forms, and that its responses to stimuli of various sorts were most simply understood upon what may be called the 'Oxford' view of cutaneous sensation. This view supposes that the different cutaneous sensations are determined by different discharge patterns in the same fibres of the nerve rami leading from the skin, rather than that different fibres in the rami are selectively activated by different stimuli. A sympathetic criticism of this view has recently been given by Rose and Mountcastle (1959).

Lele, Palmer, and Weddell point out (p. 438) that there is some evidence (not from amphioxus) that morphologically indistinguishable unencapsulated erve-terminals in the skin may be in some way differentiated to enable hem to respond to specific stimuli. The observation that free nerve-terminals n the skin may belong to different types of central neurone is of particular nterest in this connexion. It is by no means impossible that these different eurones may possess free endings with different thresholds for different ypes of stimuli. The 'C' fibres in mammalian skin which presumably ramify n free endings can be activated by cold, warmth, and mechanical stimuli, s well as by harmful stimuli (Rose and Mountcastle). It is not improbable at individual 'C' fibres may be modality-specific, if their cells of origin are s physiologically diverse as must be those of the different types which give se to the free terminals in amphioxus skin.

Probably the eventual conclusion as to the merits of the 'Oxford' and the lassical views of cutaneous sensibility will incorporate aspects of both, but t present we may conclude that the condition of the innervation of the inteument in amphioxus does not provide unequivocal support for the 'Oxford' iew. Rather, it raises the question whether good evidence for that view is rovided by the responses to stimuli of portions of integument containing nly free nerve-endings (e.g. the cornea), for it has not yet been sufficiently onsidered that these free endings may belong to very different central cells, nd may, in fact, possess different physiological attributes, while morphogically they are indistinguishable.

I am indebted to Professor C. F. A. Pantin and to Dr. M. Cowan for elpful discussion of several points.

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